Effects of secretions of the mucus gland of *Sirex noctilio* on biochemical systems in *Pinus radiata*, and some physicochemical properties of the mucus

by

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Except as stated herein, this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and that, to the best of my knowledge and belief, this thesis contains no copy or paraphrase of material previously published or written by another person, except when due reference is made in the text of this thesis.

(L.K. Wong)

PREFACE

A study of the effects of Sirex noctilio mucus on the biochemical and physiological systems of Pinus radiata, together with elucidation of some of the properties of Sirex mucus, is presented in this thesis. The first awareness of Sirex in Tasmania dates back to 1952 when Sirex larvae were discovered in baulks from the Pittwater Plantation, and when large numbers of pine trees at the plantation were destroyed by the wasps.

Radiata pine is a softwood of considerable economic importance to Tasmania, and its timber is used as pulpwood and for such domestic purposes as the making of furniture, floor boards and superstructures of houses, and packing cases. The destructive effect of Sirex larvae, manifest in the formation of extensive tunnels in the wood, render the timber useless and therefore impose a threat to the softwood industry. Where the larvae have not successfully hatched from the eggs and tunnelled into the tree, activities of the symbiotic fungus or the ovipositing wasp often result in malformations of the tree trunk, thereby reducing the value of the timber. As a result of this threat to Australia's softwood industry, studies were embarked upon by three different institutions, for the purposes of curtailing the activities of the wasp and the fungus, and for the breeding of resistant trees. The Waite Agricultural Institute at Glen Osmond, South Australia, the Entomology Division of the C.S.I.R.O. at Hobart, and the Forest Research Institute at Hobart, became involved with the fungus, the insect, and the tree, respectively. However, the decline in incidence of Sirex attacks from the late 1960's led to the dwindling of research efforts in these fields.

During the height of *Sirex* activity, plantation trees which had received a heavy attack turned yellow and dry, then died. To the casual observer it may appear that the act of ovipositing by the female wasp, and subsequent destruction of the wood by the larvae, are the causes of tree death. Studies by the Forest Research Institute have shown that each act of ovipositing is accompanied by the deposition of a secretion from the reproductive system of the wasp. This secretion, derived from the mucus gland, was identified as the causative agent in early tree death. What is the nature of *S.noctilio* mucus, and what are the mechanics of its effects?

In Part I of this thesis an account of the life history of *Sirex noctilio* is given, together with experimental observations on the physiological and biochemical responses of *Pinus radiata* foliage to *Sirex* mucus treatment. A literature survey on natural plant senescence is also presented to draw attention to similarities between the *Sirex* syndrome and the processes of natural senescence.

Studies on the physical and biochemical properties of *Sirex* mucus are dealt with in Part II. Whilst the biochemical mysteries of the polysaccharide - protein complex in *Sirex* mucus have not been delved into to any great detail, it was apparent from external appearance and from simple chemical tests, that the sticky mucus possesses properties of a "glycoprotein" and/or a "mucopolysaccharide". These two biochemical terms are used to describe groups of polysaccharide - protein complexes which have different properties, due to differences in their chemical constituents. Their definitions are generally based on material of vertebrate origin, and have been adapted for material of invertebrate origin.

The secretion of the mucus gland of S.noctilio, like the secretions and tissues of most other insects, is composed of a mixture of polysaccharide - protein complexes. The extracellular secretions of some fungi and bacteria which induce senescence in their host plants, have also been identified as complexes of polysaccharide and protein. Compatibility between these microbial parasites and their host plants is quite specific; such a relationship is also apparent amongst the siricids and their host tree species.

The basic raw material upon which studies are made in this thesis, is S.noctilio mucus. In order to meet the requirements for experimental purposes, the mucus had to be obtained from insects which were specially reared in captivity in the insectaries. Enormous costs were involved in the production of mucus, and consequently, most of the studies had to be carried out on a semi - micro scale.

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ABBREVIATIONS

AB	=	alcian blue
ABP	=	aminobiphenyl
AHP	=	aniline hydrogen phthalate
AM	=	autoclaved mucus
for aminoacids	=	refer to Appendix VIII
AO	=	acridine orange
BEW	=	n-butanol : ethanol : water
bpb	=	bromophenol blue
BPW	=	n-butanol : pyridine : water
CPC	=	cetylpyridinium chloride
CSC	=	chondroitin sulphate C
EAW	=	ethylacetate : acetic acid : water
fuc	=	fucose
gal	=	galactose
glu	=	glucose
man	=	mannose
αNA	=	α-naphthylacetate
αNP	=	α-naphthylphosphate
pNPP	=	p-nitrophenylphosphate
O.D.	=	optical density
P.A.S.	=	periodic acid - Schiff reagent
PCMB	=	p-chloromercuribenzoate
rham	=	rhamnose
TB	=	toluidine blue
TLC	=	thin layer chromatography
V_o	=	void volume

SUMMARY

The mucus secretion of S.noctilio induces a series of degradative changes in the tissues of P.radiata needles, which result in chlorosis and desiccation, and ultimately death. Dramatic changes are recorded in the respiratory rate and mode, and activities of amylase and peroxidase, in tissues which develop severe symptoms. These changes are associated with the destruction of chloroplasts, excessive loss of moisture and necrosis of food conducting elements. Basic similarities between changes caused by S.noctilio mucus, and natural and induced senescence of other plant foliage are discussed. It is also suggested that plant hormones confer a degree of resistance to the effects of Sirex mucus.

Some physiological and biochemical changes (e.g., respiration and chlorophyll) are almost immediate upon treatment of P.radiata with autoclaved mucus. This is partly due to the faster uptake of autoclaved mucus solution which is also less viscous than raw mucus solution.

Raw mucus has an estimated molecular weight of 60,000 - 100,000. In an aqueous solution, it undergoes slow spontaneous disaggregation with loss of viscosity to yield dialysable subunits of molecular weight 2,000 - 6,000, which are physiologically active. Rapid disaggregations are brought about by treatment with moderate concentrations of NaCl, KCl, or $CaCl_2$, or by heating, but not with urea. The presence of amylase, esterase, acid phosphatase, phenoloxidase and proteolytic enzyme activities in native mucus may be partly responsible for the process of "natural" disaggregation.

The mucus appears to be a fairly homogeneous polysaccharide - protein complex with properties of both a neutral and an acid polysac-

charide. The acidic polysaccharide bears close similarities to mammalian hyaluronic acid in its reactions to histochemical stains. Thus, it reacts with basic dyes at pH 3 - 7, it is orthochromatic in toluidine blue, red in acridine orange, blue - green in alcian blue, and its reactions with alcian blue appears to be mediated through its carboxyl groups, however, it shows high resistance to mammalian hyaluronidase.

Two major polysaccharide - protein complexes, both with physiological activities, are separated from DEAE - Sephadex, using autoclaved mucus. The first complex is eluted in 0.1 - 0.4 M NaCl, and appears to be a collageneous type of sulphated glycoprotein, consisting mainly of fucose, galactose, glucosamine and galacturonic acid. The second complex is eluted in 0.5 - 0.9 M NaCl, and appears to be a conjugate of sulphated glycoprotein and hyaluronate type of acid mucopolysaccharide. A total of five hexoses (i.e., rhamnose, fucose, mannose, glucose and galactose), two hexuronic acids (i.e., glucuronic acid and galacturonic acid), and two hexosamines (i.e., glucosamine and galactosamine) are present. The mucus secretion of S.noctilio is compared with extracellular secretions of microbes which also induce senescence of their host plants. A brief study of mucus secretions from two other siricids, i.e., Urocerus gigas and Xeris spectrum, reveals the presence of protein and carbohydrate, but these secretions do not induce foliar senescence of P.radiata, and probably indicates a high specificity of wasp to host tree species.

PART I

Effects of secretions of the mucus gland of *Sirex noctilio* on biochemical systems in *Pinus radiata*

GENERAL INTRODUCTION

History of Sirex noctilio

The attention and subsequent research into the Sirex problem in Australia was due first to the discovery of borers in baulks from the Pittwater Plantation in Tasmania in March 1952, and to the extensive destruction of pine plantations at Pittwater. The insect was believed to have been introduced from Europe not later than the summer of 1950/51 from infested timber or timber products (Gilbert and Miller, 1952).

Pittwater is located 19.2 Km east-north-east of Hobart. Since its discovery there, the wasps spread especially towards the north and north-west of Tasmania. The height of Sirex infestation was reached in 1959, when it was found in almost any considerable stand of Pinus radiata (Mucha, 1967); however the occurence of the wasp and its destructive effect on pine trees had since declined. Figure 1, taken from Mucha (1967), shows the occurence of Sirex noctilio in areas of Pinus radiata in Tasmania from 1950 - 1965. The rapid spread of Sirex prior to 1959 was thought to have been aided by man through the transportation of infested timber from one area to another, rather than wholly through a natural process of insect dispersal.

In Victoria, S.noctilio was discovered at Woori Yallock, 48 Km east of Melbourne, in December 1961. The wasps were subsequently found over an area of more than two million sq. Km around Melbourne, especially towards the north and east within 7 years of its discovery there.

S.noctilio was introduced into New Zealand in infested timber from Europe before 1900. Its effects were not felt until the epide-

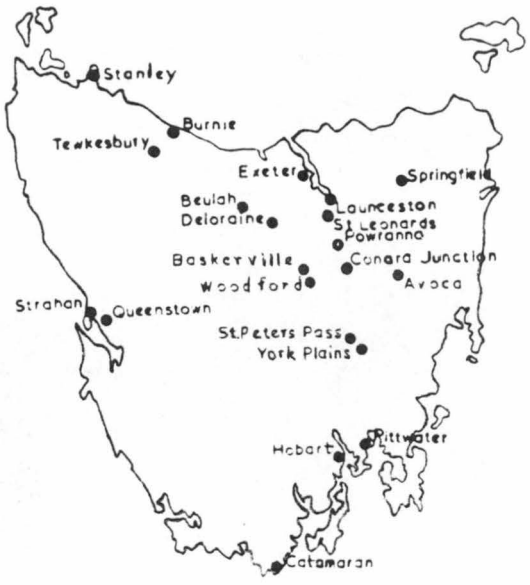

Fig. 1. Names and location of places

Fig. 2. White indicates radiata distribution throughout Tasmania.

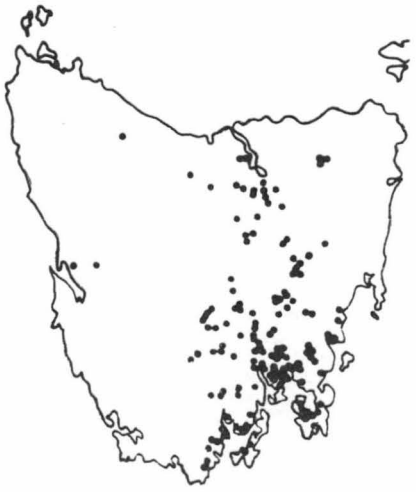

Fig. 3. Black dots indicate Sirex noctilio occurrences, 1950/65

Fig. 1: Maps of Tasmania showing

1. Names and location of places
2. Distribution of Pinus radiata
3. Distribution of Sirex noctilio

(Taken from Mucha, S. (1967) -- The establishment and spread of S.noctilio F. in Tasmania from 1950 to 1964. Australian Forest Research, Vol. 3(1), pages 3 - 23).

mic of 1948 - 52, when large areas of P.radiata in the Rotorua district were destroyed. Prior to this epidemic, the forests were overcrowded, and had received practically no silvicultural treatment. The resulting poor state of the forests, together with a period of drought, provided ideal conditions for an outbreak of Sirex. Mortality was confined to malformed and suppressed trees in the overcrowded stands, and as such became beneficial as the Sirex outbreak had helped to ease the overcrowded situation in the pine forests by removing trees of poor economic value (Rawlings and Wilson, 1949).

Distribution of siricids

The family Siricidae consists of three subfamilies with 100 species and subspecies. These occur as indigenes in the Palearctic, Nearctic and Oriental regions of the world. The subfamily Siricinae consists of one extinct and six extant genera; it includes Urocerus gigas and Xeris spectrum which have subspecies in Asia, Europe and North America; Sirex areolatus and Sirex cyaneus which are both Nearctic species established in Europe; and Sirex juvencus and Sirex noctilio which are Eurasian species established in Canada (Morgan, 1968). Some endemic species of restricted distribution are Sirex imperialis of north India, Sirex mongolorum of Mongolia, and Sirex nitobei of Japan (cited in Gaut, 1970).

Biology of Sirex noctilio in Tasmania

Adult wasp

Male and female wasps exhibit readily recognisable external differences. The male wasp has an orange coloured abdomen with stout short black hind legs and no ovipositor. The female wasp has a metal-

lic black abdomen, thin slender yellow hind legs and a long ovipositor adapted for boring. Size variation is common within and between the sexes.

The anatomical arrangement of the reproductive system of the female wasp, taken from Boros (1968), is given in figure 2. Associated with the reproductive system are three pairs of accessory glands, being the lateral pouches, the oil sacs and the mucus glands which open into a common mucus reservoir. On the basis of their similar anatomical arrangements, the mucus glands and reservoir, the oil sacs and the lateral pouches of S.noctilio are thought to be homologous to the poison sacs and glands, the alkaline glands and the lubricating glands, respectively, of the honey bee (Boros, 1968). A pair of club glands located at the base of the first pair of valvulae, contain the arthrospores of the symbiotic fungus.

Suitable sites for oviposition on the tree trunk are selected by means of the antennae, and multiple tunnels are usually drilled at each site. The number of tunnels which are drilled at each site depends on the moisture content of the tree. At extreme moisture conditions single tunnels are drilled, but when moisture conditions become more intermediate, the proportion of single tunnels decrease with an accompanying increase in the number of triple tunnels. At these intermediate moisture conditions, there is a very high proportion of double tunnels (Coutts, 1965).

During ovipositions, a mixture of oil and mucus secretion is deposited into the tunnels along with eggs or fungal arthrospores. The eggs and arthropsores are laid in separate tunnels, although single tunnels usually do not contain eggs (Coutts, 1965). The oil

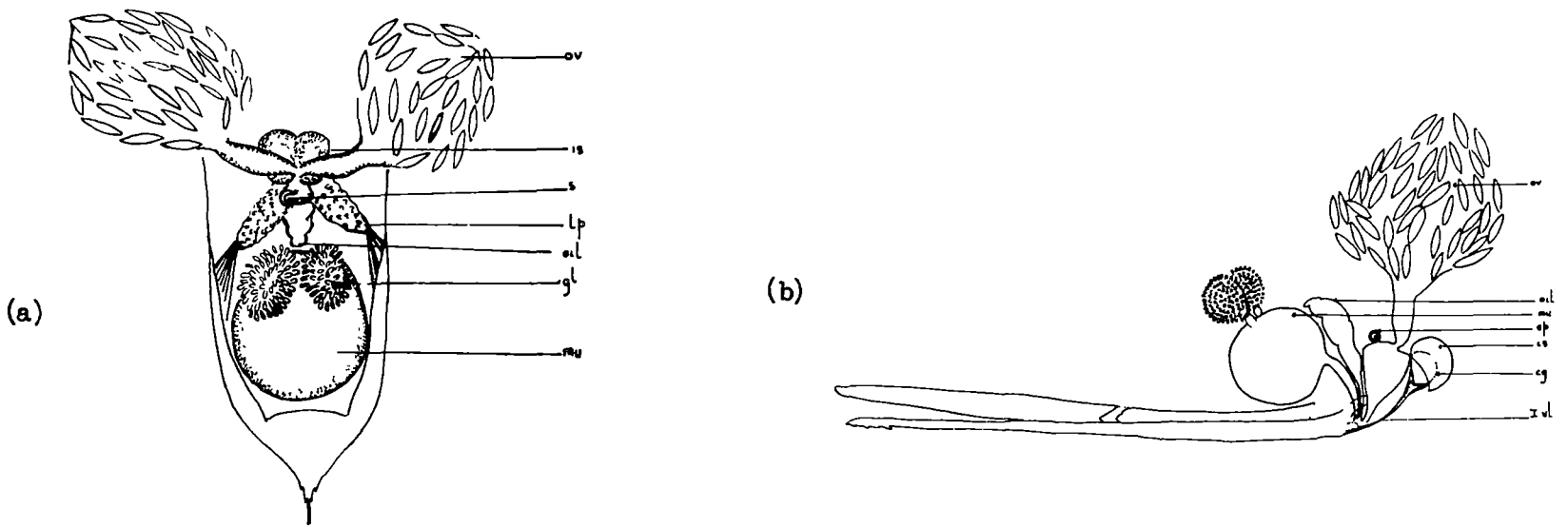

Fig. 2. Reproductive system of adult female Sirex noctilio; dorsal (a), and lateral (b), views.

mu gl	= mucus gland	sp	= spermatheca	is	= intersegmental sac
oil	= oil gland	od	= oviduct	cg	= club gland
I vl	= first valvula				

(These figures, at half of the original size, have been reproduced with the kind permission of Mrs. Boros, from her thesis: Boros, C.B. (1968) — M. Sc. Thesis, University of Adelaide, South Australia.)

and mucus secretions serve as a lubricant for the insect ovipositor, and also provides a growth medium for the fungus (Boros, 1968).

The period of incubation of S.noctilio eggs depends on the tree moisture content and on the ambient temperature. In Tasmania, eggs hatch in two weeks in the warm summer weather; in New Zealand, eggs hatch in 16 to 28 days.

Larva and pupa

The Sirex larva has a whitish body with three pairs of vestigial thoracic legs. Female larvae are distinguished from male larvae by the presence of a pair of hypo-pleural organs located at the first abdominal segment, and two small sclerites on the terminal abdominal sternite, of the female larva. Male larvae have no hypo-pleural organs but contain three terminal sclerites. The hypo-pleural organs consist of a series of pits within which are borne wax packets containing the fungal arthrospores (Boros, 1968).

The larvae depend on fungal mycelia for nutrition. In studies of larvae of Sirex juvencus and Urocerus gigas, Francke-Grosmann (1939) found that the digestive juices of these larvae had no effect on cellulose, hemicellulose or wood fibres, but readily digested the fungal mycelia. Further evidence that the wood was not digested by the larvae was presented by Muller (1934) when he found little chemical differences between the frass in the larval tunnels and the surrounding unchewed wood of the tree.

The number of ecdyses and the length of larval existence are dependent on the availability of food, tree moisutre content and ambient temperature. Under favourable conditions, there is a tendency for larval life to be prolonged.

Extensive tunnels are formed in the tree by the burrowing larvae, by chewing the wood ahead and leaving behind frass packed tunnels. Although there is no definite polarity of movement by larvae in the host tree, they often burrow into the heartwood and then return to the periphery of the trunk for pupation. Pupal chambers are formed close to the bark of the tree and pupation lasts for about a month. Completion of the life cycle within the host tree takes from one to two years. Adults emerge from circular flight holes during late November to early May, with the height of activity occuring in February to March.

Parthenogenisis is common amongst Hymenopterans; unfertilized eggs develop into male progeny whereas fertilized eggs develop into female progeny. As a result, the overall male population of S.noctilio tends to be larger than that of females. The male : female ratio is estimated to be 9:1 (Madden, et al, C.S.I.R.O. Annual Report, 1964/1965).

Male wasps tend to emerge from pupation earlier than female wasps. They have less desire to disperse from the area of emergence than female wasps, and usually congregate around the tree tops in the area of emergence. Female wasps usually disperse from the area of emergence before being mated, and then enter into a phase of oviposition which culminates in death.

Fungal symbiont of Sirex noctilio

Fungal symbionts associated with Sirex wasps belong to the class Basidiomycetidae. They do not form fructifications in nature, and are wholly dependent on the wasps for transmission from one host tree to another. The mode of reproduction is asexual, with the formation

of arthrospores through fragmentation of vegetative hyphae. The number of nuclei in each arthrospore is found to vary from one to six (Gaut, 1970). The arthrospores are contained in the hypo-pleural organs of larvae and in the intersegmental sacs of adult female wasps.

The S.noctilio fungal symbiont was identified as a species of Amylostereum by Talbot (1964), from fructifications of fungal cultures produced on wood blocks. King (1966) compared the S.noctilio fungus from Australia with Amylostereum chailletii (Fr.) Boidin from Canada, and found close similarities in their electrophoretic protein patterns, thus suggesting that the Australian S.noctilio fungal symbiont may be a strain of A.chailletii. However Gaut (1969) identified the Australian S.noctilio fungal symbiont as Amylostereum areolatum (Fr.) Boidin on the basis of five criteria, which involved the use of such techniques as the isolation of homokaryons, anastomosis, dikaryotization and interfertility tests, and comparisons of their protein patterns after starch gel electrophoresis. Moreover, laboratory cultures of A.areolatum produced arthrospores whereas those of A.chailletii failed to produce any.

In his survey, Gaut (1970) suggested that a species - specific relationship exists between the wasp and its fungal symbiont, so that any one siricid species is associated with a particular species of fungus, regardless of their geographical distribution. There were two anomalies which Gaut thought were due partly to incorrect identification of the Sirex species. Thus, A.areolatum is associated with S.juvencus, S.nitobei and S.noctilio, whilst A.chailletii is associated with S.areolatus, S.cyaneus, S.imperialis, S.californicus, Urocerus augur augur, Urocerus augur sah and Urocerus gigas gigas. Compa-

risons of protein patterns on starch gel electrophoretograms showed that the S.noctilio fungal symbiont from Australia had close similarities to the Belgian, Swiss and Japanese isolates, which suggested similar geographical origins. Gaut also recognised two basic groups of fungal protein pattern, one of which comprised the fungal isolates from Switzerland, Belgium, Britain, Japan and Australia, the other comprising the fungal isolates from France, Portugal and Morocco.

Amylostereum causes white rot in pine trees, which results in the rapid decay of these affected trees in New Zealand, but not so under Australian conditions. The fungus spreads in a longitudinal direction and dries the wood ahead of it thereby causing a white discoloration. Although the xylem tracheids are not occluded by the fungal hyphae, the ray parenchyma cells of the wood are killed (King, 1966).

Amylostereum was isolated from the phloem and cambium of infected P.radiata tree trunks. The fungal hyphae were abundant and widespread around the tunnels during the period of larval development, gradually declining but persisted in the year following the emergence of adult wasps. With the decline in growth of Amylostereum other fungi became abundant, namely Macrophoma sabinea and especially Trichoderma viride (Vaartaja and King, 1964). Macrophoma sabinea causes an intense blue staining of the tree, whilst Trichoderma viride has been used for the control of Fommes annosus, a basidiomycete (Rishbeth, 1959). Vaartaja and King (1964) also isolated eight other genera of fungi from trees at various stages of Sirex infestation.

In laboratory cultures, glucose and sucrose were found to be

the most suitable carbohydrate substrates for the growth of Amylostereum. When fatty acids were used as carbon substrates, the suitability of the substrate for growth tended to increase with increasing carbon chain length of up to C-18. Fatty acids containing more than 18 carbon atoms became increasingly less suitable (Gaut, 1970).

Host trees

Softwoods, usually conifers, form the main type of host for members of the subfamily Siricinae. In Australia, Pinus radiata (Gymnospermae: Coniferales) is the main host for S.noctilio. In addition to P.radiata, twelve other species of Pinus were killed by S.noctilio in New Zealand, and successful development of Sirex from moribund branches of Larix and Pseudotsuga were also recorded (Morgan and Stewart, 1966). There were sightings of Sirex ovipositing in rimu, miro, kauri and Eucalyptus globulus but development of the wasp from these trees was unsuccessful (Rawlings, 1948).

P.radiata is a native conifer of California, where it grows in isolated pockets along the coast and coastal ranges and is subjected to attack by mistletoe and numerous species of insects. It was introduced into Australia and New Zealand by the early colonists, and has since thrived and established itself. It is an important source of softwood timber for use in domestic purposes. There are over 50,000 acres of forests in Australia, and more than half of this consists of P.radiata.

Parasites and predators of S.noctilio

In Tasmania, the native Ichneumonid, Certonotus tasmaniensis (Ichneumonidae: Labenini) is an ectoparasite of Sirex larvae. It

lays its eggs on the surface of small Sirex larvae which are found within $\frac{3}{4}$" of the tree surface, due to a short ovipositor. Its activities are confined to smaller wood lots and it has an earlier flight season than Sirex. Certonotus is of sporadic occurence in Tasmania in localities where native tree species are found in the vicinity of P.radiata stands, and it is absent from the Pittwater Plantation. It is not considered to be likely to contribute significantly to the biological control of Sirex (Hocking, 1967).

Various species of Ibalia (Hymenoptera: Ibaliidae), Rhyssa (Hymenoptera: Ichneumonidae) and Megarhyssa (Hymenoptera: Ichneumonidae) have been introduced into Australia and New Zealand for the purpose of controlling S.noctilio. Some of these insects include R.persuasoria, I.leucospoides, I.drewseni and M.emarginatoria from Europe and the United Kingdom; R.himalayensis from India; and M.nortoni nortoni, R.alaskensis and I.ensiger from California. The insects have been reared at local insectaries and liberated into the Sirex infested areas.

The biology and behaviour of Ibalia leucospoides, Rhyssa persuasoria, and Megarhyssa nortoni nortoni have been studied in detail by the Entomology Division of the C.S.I.R.O. in Hobart. Ibalia lays its eggs in the eggs or newly hatched larvae of Sirex. Rhyssa and Megarhyssa lay their eggs in the frass packed tunnels and sometimes close to the host larvae. Active competition between these three genera is not apparent as Ibalia oviposits earlier than either of the two genera, and Megarhyssa which possesses a longer ovipositor is able to reach the Sirex larvae that are found deeper in the wood of the host tree. There is no evidence of hyperparasitism.

Ibalia usually oviposits when the oviposition drills of Sirex are still very moist and contain a high amount of volatile oils. Rhyssa and Megarhyssa oviposit when the wood moisture and volatile oil contents are lower. The presence of actively growing fungus is believed to be the source of attractant to the insect parasites, rather than the presence of Sirex eggs (Madden, 1968). Although the actual chemical attractant has not been identified, aqueous, methanol, ethanol and acetone extracts of fungal cultures and of frass have produced positive bioassay results. Bioassay was based on antennal and ovipositor responses from female insects. Each of the three genera of insect parasites studied, was found to respond to different threshold levels of extracts, which probably resulted in their exploiting hosts at differect depths in the tree trunk. These extracts also induced the display of courtship activity in male M.nortoni nortoni.

Since their introduction into Tasmania, the overall number of insects which emerged from Sirex infested logs had been falling relative to the Sirex numbers. The C.S.I.R.O. reported that Ibalia numbers had declined from 31.1% in 1963/64, to 17.7% in 1964/65, and to 6.9% in 1965/66. The fall in numbers was partly attributed to the "unusual" climatic factors over these three seasons, as a result of which Sirex emerged in two peak periods, with the Ibalia emergence coinciding with the first peak of Sirex emergence. The proportion of Rhyssa relative to Sirex numbers remained fairly stable during the same period.

Schlettererius cinctipes (Hymenoptera: Stephanidae) collected in the vicinity of siricid infested Pinus jeffreyi Murr. and Abies spp. in California, was introduced into Tasmania. S.cinctipes eggs are

laid on the surface of Sirex larvae, and when hatched become ectoparasites. Like Ibalia, Rhyssa and Megarhyssa, Schlettererius wasps have been successfully bred from Sirex infested logs in the insectaries.

At the Pittwater Plantation, twelve species of birds were seen to prey upon Sirex wasps and of these, three bird species preyed on Ibalia and at least two bird species preyed on Rhyssa. The dusky wood swallow Artamus cyanopterus was the most abundant and active predator (Madden, et al, 1964/65 C.S.I.R.O. Report).

Zondag first reported on the infestation of a Sirex population with nematodes, from a Pinus patula stand in New Zealand in 1962. The nematodes were later identified as a species of Deladenus (Neotylenchidae) (Bedding, 1967), and found to be present in both the adult and larval stages of Sirex. In the adult hosts, nematodes were usually confined to the reproductive organs where they caused hypertrophy of testes or atrophy of ovaries. Female wasps infected with nematodes continued to oviposit, unimpeded.

Consignments of R.himalayensis from India, R.persuasoria from Europe and U.K., and a few individuals of R.amoena from Europe were found to be infested with nematodes (Deladenus sp.) on arrival in Tasmania. There were some apparent differences in the pathological effects and life history of the nematodes which infested Rhyssa and Sirex (Hocking, 1967). In Rhyssa, the nematodes occured free in the body cavities of their hosts where they caused damage to the alimentary system and to the reproductive organs of females, although the male reproductive organs remained unaffected. Sirex wasps in Victoria and Tasmania have not been naturally infested with nematodes,

and of those nematodes which infested R.persuasoria, oviposition by these wasps resulted only in infested Rhyssa larvae without affecting the Sirex larvae.

Since the discovery of nematodes in S.noctilio from New Zealand, other siricids found to be also infested with nematodes were S.juvencus, S.cyaneus, U.gigas and X.spectrum. The nematodes which infested the siricids and their hymenopteran parasites belong to two species of Deladenus (Bedding, 1967). Two forms of nematodes are produced from each of these species, dimorphism being most marked in females. The common form of nematode is non-parasitic and feeds on the mycelia of the fungal symbionts of insects. Copulation takes place, and eggs produced from these non-parasitic females develop into male and two types of female larvae.

The less common form of nematode is parasitic, it does not feed on fungal mycelia, does not copulate and is viviparous. Young larvae produced by these parasitic nematodes enter the haemocoel of their host larvae, and continue development there. At the stage when the hosts complete pupation and emerge from the tree as adults, the infective nematodes produce their young larvae which then consists of males and the two forms of females.

Morphological differences between the two forms of females are distinct, and although not so obvious in males, the two forms of male nematodes produced distinctly different types of sperms. Bedding (1967/68, C.S.I.R.O. Report) identified the siricid - infesting nematodes as Deladenus siricidicola and the rhyssine - infesting nematodes as Deladenus wilsoni. Both D.siricidicola and D.wilsoni occasionally enter Ibalia spp. but they fail to develop in these hosts.

Control of S.noctilio

Biological

Insect predators, native or introduced into Tasmania, have been assessed in their potential for use in Sirex control. The sporadic occurence of Certonotus tasmaniensis in Tasmania, and its natural physical limitations make it unsuitable for Sirex control (Hocking, 1967). Ibalia, Rhyssa, Megarhyssa and Schlettererius are genera which were introduced into Tasmania and New Zealand. These insects have been successfully propagated at local insectaries and liberated into Sirex infested areas. On their own, it is doubtful if they can effectively control S.noctilio populations, when edaphic conditions become favourable for Sirex development (C.S.I.R.O. Report). However, when used in conjunction with silvicultural practices, the insect predators may be effective in controlling the increase in Sirex numbers at any one particular area.

Various species of birds have taken their toll of both S.noctilio and its insect predators at the Pittwater Plantation. Effects of avian predation have not been viewed as drastic.

Infestation of S.noctilio by nematodes in New Zealand, and also of Rhyssa spp. from overseas, reduce the fertility of the insects and viability of their eggs. Nematodes have not been released in sufficient numbers for an assessment of their effects to be made.

Chemical

Sirex wasps were found to be tolerant to high doses of contact insecticides like D.D.T., Gammexane, Toxaphene and Rothane (Rawlings and Wilson, 1949), though several organic phosphates and one carbamate were found to be effective (Horwood, 1966, as quoted in Morgan, 1968).

Fumigation of infested timber with methyl bromide is a relatively cheap and convenient method but inconvenient because of the exceptionally high degree of resistance of Sirex larvae to this chemical, and of the unsatisfactory distribution of the fumigant in close - piled timber (Boocock, 1959). Pressure impregnation of timber with Boliden, Celcure and Tanalith salts at a temperature of at least 41°C, though effective and economically feasible for the treatment of better quality timber such as those used for the building industry, is too expensive for the treatment of timber designated for packing cases.

The spraying or injection of systemic fungicides into Sirex infested trees failed to control the fungus, due to the poor diffusion of fungicide into the tree and to the characteristic mode of transportation of the fungicide, i.e., spiral transportation with little or no lateral transportation, so that only a strip of fungus was effectively killed (Stahl, 1965).

Heat

Heat sterilization of individual infested timber by means of steaming for 10hr at 74°C is an effective and reliable means of larval control. Kilning is also a suitable method but economically unattractive because of the requirement for extra kiln space and the high costs involved in handling and treatment.

Silviculture

Maintenance of healthy trees by sound silvicultural means is the key towards effective Sirex control. Rawlings (1948) cited two cases in New Zealand where heavy mortality of P.radiata due to Sirex

infestations was incurred following heavy thinning and pruning of the trees. One of these was an area of 9 year old regeneration forest at Waiotapu, and the other of 20 year old stands at Kainga-roa. In both cases, the trees had poor crown development and were severely thinned and pruned in the warm weather during the Sirex flight season, which resulted in the trees being suitable material for Sirex development. In view of these observations, the silvi-cultural measures suggested by Rawlings (1948) included frequent light thinnings and maintenance of good crowns of more than one third of the total height of the trees, during periods when the Sirex wasps were not in flight, and the removal of weak or damaged trees. It was noted that trees with poor crown development, i.e., those with less than one quarter of their height in crown, were more liable to successful Sirex attack and development than those with crowns exceeding one third of the total height.

Whilst the above-mentioned silvicultural measures are usually undertaken in state and privately - owned pine plantations, there is need for private property owners who grow pine trees as wind-breaks to co-operate. Damaged or moribund branches of old pine trees should be removed from their properties, as these serve as suitable sites for Sirex development.

In Victoria, "trap trees" placed in areas where no "natural" Sirex attacks were previously recorded, were found to attract Sirex ovipositions (Madden, et al, 1964/65, C.S.I.R.O. Report). It was felt that the use of such "trap trees" together with contact insec-ticides may be effective in keeping S.noctilio under control, in limited areas of forests (Morgan, 1968).

Responses of P.radiata to the insect and the fungus

Trees which are suppressed, moribund or weakened by physical damage or fire become highly attractive to Sirex wasps. The 1948 - 1962 Sirex epidemic in New Zealand was caused by severe overcrowding of trees and the outbreak had occured during the period of drought, during which trees of the suppressed class were killed.

Suppressed trees do not exhibit vigorous growth rates and Madden (1968/69, C.S.I.R.O. Report) found that the naturally attacked trees suffered impaired cambial activity. Buds of susceptible trees also had lower phenoloxidase and peroxidase activities, but there were no differences in activities of phosphatase, phosphorylase and tannin content between the susceptible and resistant trees, on the basis of histochemical staining (Titze, 1965).

Unhealthy trees become suitable for Sirex development as a result of an altered mode of metabolism. Titze (1965) suggested that suppressed trees which produced more fats and fatty acids than carbohydrates were more suitable for Sirex development. Pruned, ringed or "bark cinctured" trees attracted Sirex ovipositions to the starved areas, and these methods essentially prevented the translocation of photosynthates to the area below or within the debarked regions (Coutts and Dolezal, 1966a; Madden, et al, 1964/65, C.S.I.R.O. Report). The latter recorded a lag of 10 - 12 days before the "starved" end of the ringed tree became attractive to Sirex.

Dominant trees have a higher moisture content in the outer half inch of wood than suppressed trees, and there is a tendency for the moisture content to increase with tree height, in both cases. The moisture content of the tree affected the drilling behaviour of the

wasps, and the survival rate of their larvae (Coutts, 1965; Coutts and Dolezal, 1965). In logs of high moisture content, survival rate of the larvae was low, but in logs of low moisture content the eggs and larvae were caused to dry out. Thus small trees felled in spring and summer prevented successful development of Sirex because of the rapid rate of drying out, but trees felled in autumn became suitable for Sirex development (Wolfe, 1966). Within two days of burning, the scorched areas of trees also attracted Sirex ovipositions.

Resin exudation from oviposition sites is one of the early symptoms of Sirex attacks. Coutts and Dolezal (1966b) found that at any one oviposition site, where double tunnels were drilled, the longer tunnel containing fungal arthrospores became almost filled with solidified resin three days after oviposition, and the shorter tunnel containing the egg was more or less free of resin. However, eggs and larvae became trapped and killed when resin production was copious, and female wasps exhausted from ovipositing also became trapped with their ovipositors still inserted into the tree trunk, by the copious resin flow. Titze and Mucha (1965) suggested that a high resin flow is one of the immediate means of tree defense against Sirex attack. The successful attack of Araucaria cunninghamii by the black pine weevil Vanapa oberthuri was associated with poor resin flow in the bark (Barrett, 1967).

The fungus spreads in a longitudinal direction from the site of infection and dries the wood ahead of it. Tree polyphenols first detected in the outermost growth ring within the fungus - dried areas of wood, two weeks after oviposition, serve to contain the growth and spread of the fungus, thereby giving rise to areas of red - stained

wood (Coutts and Dolezal, 1966b). In bark cinctured trees, the starved region of the trunk which supported a good growth of Amylostereum was found to have a low polyphenol content, whereas the untreated, healthy area of the trunk which had a high level of polyphenol allowed only a sparce growth of the fungus.

The major phenolic compounds which were formed in P.radiata following a Sirex attack, were identified as pinosylvin and pinosylvin monomethylether, i.e., trans-3,5-dihydroxy-stilbene and 3-hydroxy-5-methoxy-stilbene, respectively (Hillis and Inoue, 1968). In P.resinosa these pinosylvins are restricted to the heartwood, they are absent from normal undamaged sapwood, and they are present in the wounded sapwood. Under laboratory conditions, sapwood cells which were killed rapidly did not form pinosylvins, but those which were allowed to die slowly under conditions of desiccation and, or, aeration, formed pinosylvins (Jorgensen, 1961). It was concluded that the pinosylvins were formed by the living sapwood as a defense reaction against wounding and pathogenic invasion. These polyphenolic compounds act as uncoupling agents which inhibit oxidative phosphorylation, the main source of energy of decay fungi (Scheffer and Cowling, 1966). Ethylene was also produced in the sapwood of P.radiata following mechanical injury or Sirex attacks (Shain and Hillis, 1972). Those trees which produced more ethylene also produced more polyphenols, and the production of ethylene preceded that of polyphenols.

The cause of tree death was thought to be the result of fungal growth, which interrupted with the sap supply to the crown (Rawlings, 1948). Fungal hyphae did not occlude the xylem tracheids but killed the ray parenchyma cells of the wood (King, 1966). Coutts (1968)

demonstrated that healthy trees did not turn chlorotic when they were mechanically damaged to the extent of injuring much of the sapwood. Instead, water extracts from logs previously attacked by Sirex wasps, if injected into healthy trees, caused the development of symptoms of natural Sirex attack. Extracts of logs previously hand - inoculated with Amylostereum alone, did not produce the Sirex symptoms, when injected into healthy trees. Similarly, there was only a slight development of some of the symptoms of Sirex attack in the area immediately surrounding the inoculation site, when suspensions of fungal mycelia were injected into healthy trees (Titze and Stahl, 1970).

Young P.radiata seedlings with intact roots, wilted without turning chlorotic when they were treated with aqueous solutions of acebone or ethanol precipitates of Amylostereum culture filtrates (Titze, 1970). Chemical tests revealed the presence of protein and reducing sugars in these solutions, and Titze assumed that a protein - polysaccharide complex produced by Amylostereum was responsible for the wilting syndrome in pine trees. The culture filtrates also contained pyruvic acid, acetaldehyde (and ethanol) but there were no other organic acids present. This implied that fungal metabolism was essentially anaerobic. In vitro experiments using fresh or boiled extracts of Amylostereum caused a 20% increase in the respiratory rate of freshly chopped P.radiata needles (Gaut, 1970).

In trees which sustain a heavy attack by Sirex wasps, the drying out of the sapwood by the fungus may restrict sap supply to the crown and cause the foliage to wilt and shrivel up. The utilization of food reserves from the host tree for fungal growth, and the extracellular secretions of the fungus, all contribute to the weakening

and eventual death of the host tree.

The observable syndrome of responses to Sirex attacks, in susceptible trees, include the progressive yellowing, drying and premature abscission of first the older and then the younger needles, and finally death of the apical shoots. Other physiological changes which occur in trees which resist or yield to Sirex attacks include a temporary cessation of growth (Coutts and Dolezal, 1966); growth only resumed if the tree was resistant. Regardless of whether the tree was resistant or susceptible, starch accumulated in the needles in the first two weeks of attack, due to active photosynthesis (Coutts, 1969a). Susceptible trees developed further symptoms by showing a reduction in the level of starch after the first two weeks; loss of starch in the needles was accompanied by similar changes to the dry weight of the needles and to the starch level in the bark of the tree trunk. Coutts (1969a) suggested that the loss of starch from the bark was due to interruption of translocation of photosynthates; however this loss could also be attributed to increased respiratory rates in the bark (Madden, 1968).

Coutts (1969b) then injected into standing trees, aqueous solutions of fresh, boiled or autoclaved mucus. The syndrome of responses in these trees were similar to those which were subjected to light Sirex attacks. Although there was great tree-to-tree variation in the degree to which symptoms were expressed, none of the trees died from treatments with fresh, boiled or autoclaved mucus. When fungal arthrospores were used in combination with mucus, trees expressed symptoms similar to those which had received a heavy Sirex attack, and died. From the experiments of Coutts, it was apparent that Sirex

mucus was the main cause of needle chlorosis. On its own, mucus did not cause tree death, but in combination with fungal arthrospores, it proved to be lethal. Coutts (1970) suggested that mucus has a physiological conditioning effect on the host tree, but of the processes which are known to occur, i.e., prevention of photosynthate translocation and disappearance of food reserves from the stem bark, chlorosis and premature abscission of the foliage, it was not clear which of these processes was the most important.

Concluding remarks

The Sirex wasp and the fungus derive mutual benefits from their association together, whereby the fungus causes the wood to dry out and helps kill the tree with its toxic secretions, besides being consumed as food by the growing larvae. The wasp acts as a vector for the transmission of the fungus from one host tree to another, and also helps to provide a more favourable environment within the tree in order to allow for successful establishment and subsequent development of the fungus, by weakening the host through initiating premature foliage chlorosis and abscission and possibly also severing the channels of photosynthate translocation to the tree trunk. This is achieved through the deposition of a secretion from the mucus gland, during ovipositions. Although heat stable, the mucus alone is believed to be ineffective in killing the tree, but when combined with fungal arthrospores, the mixture is toxic. Similarly, the fungus alone does not induce the development of "severe Sirex symptoms", although aqueous solutions of partially purified fungal extracts are known to wilt P. radiata saplings.

In response to attempts by the invading insect and fungus to weaken it, the tree counteracts by producing more resin and polyphenols. However, success or failure to contain the parasites depends largely on the health and vigour of the tree prior to the attack. Susceptibility occurs when the tree is suppressed, weak, moribund or damaged.

The observable symptoms of a Sirex attack include the premature yellowing of the older and then the younger foliage in progressive sequence, and their abscission. Other physiological changes are the reduction in manufacture of starch after the first two weeks, general cessation of growth, and reduction of starch levels in the stem bark together with stimulation of respiratory activity. These changes resemble some of the processes which occur during natural plant senescence. The causes and processes of natural plant senescence which have been reported for a variety of plants, are presented in the following chapter.

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PLANT SENESCENCE

The term senescence describes a series of progressive deteriorative processes which occur in a cell, an organ or in the whole organism, ultimately leading to the termination of the functional life of part or all of the organism. In many plants, senescence confers distinct advantages, in terms of ecological adaptation, natural selection and efficiency of internal physiology, according to seasons. Thus, weeds which adapt readily to new environmental niches are predominantly annuals which undergo overall senescence with a rapid turnover of the population and thus gain advantage in evolutionary adaptation (Leopold, 1961). Overall plant senescence is usually associated with reproduction and experiments of Murneek (1926) with tomato plants, Lycopersicon esculentum, led to the interpretation that the cause of plant senescence was the result of depletion of endogenous nitrogen from the whole plant due to mobilization to the fruits. The removal of flowers or fruits from the plants caused renewed and more vigorous vegetative growth, flower development and finally fruit formation. In Pinus resinosa Dickman and Kozlowski (1968) have shown a preferential mobilization of carbohydrates by reproductive tissues over vegetative tissues, and Leopold, et al (1959) using soybean and spinach plants have shown that the onset of senescence was effectively delayed by the removal of either flowers or fruits, with the effectiveness of such treatment declining progressively as the time for removal of the reproductive tissues was delayed. These authors suggested that overall plant senescence was a response to a gradually intensifying signal in the reproductive plant rather than depletion of food reserves through mobilization into developing organs. The

male plants of dieocious species also die after the production of staminate flowers.

There are three different types of leaf senescence depending on whether the leaf is intact on the plant, or detached (Simon, 1967). For leaves intact on the plant, the three types of senescence are progressive or sequential, synchronous and overall. The process of senescence for excised leaves held in the light is different from those which are held in the dark or in dim light.

Synchronous senescence of all leaves takes place as a result of alteration to the photoperiod or light intensity, or as a result of moisture stress. Deciduous trees respond to the shortening day-length of autumn by the shedding of yellowed leaves. Artificial lengthening of the photoperiod or application of plant growth hormones often postpones the onset of synchronous senescence.

Death, first of all of the older, and then the younger leaves in a progressive ascending order, is described as progressive or sequential senescence. This is readily observed in cotyledons or first leaves of plants which die during the early life of the plants.

The senescence of intact cucumber cotyledons (Cucumis sativus) resulted in the loss of chlorophyll, and reduction in fresh weight followed by the lowering of RNA content and nitrate reductase activity. The activities of peroxidase, glucose-6-phosphate dehydrogenase and RNAase were maintained at a high level throughout senescence (Lewington, et al, 1967). Differences were noted in the pattern of change between detached and attached cotyledons during senescence. The detached cotyledons whilst showing very rapid loss of protein and RNA, had a much faster rise in activities of peroxidase, glucose-6-phosphate

dehydrogenase and RNAase, when compared to attached cotyledons. There was a rise in the peptidase activity of senescing tobacco leaves (Nicotiana tabacum) which correlated with the fall in protein content (Anderson and Rowan, 1965). The starch content of tobacco leaves reached a maximum when more than half of the original chlorophyll content was lost, the starch content then decreased rapidly and remained at a very low level when the leaves turned yellow and brown (Matheson and Wheatley, 1962).

In the primary leaves of Phaseolus vulgaris, RNA and chlorophyll levels increased in expanding and maturing tissues, but subsequently declined during senescence. The increase of RNA in maturing leaves was accompanied by an increase in the RNAase activity. There was a simultaneous decrease in both RNA and RNAase during senescence. Phillips and Fletcher (1969) suggested that the enzyme RNAase was correlated with the rate of turnover of RNA, rather than with the absolute level of RNA present. The level of DNA remained unaltered during senescence.

Leaves of Perilla frutescens undergoing senescence lost their chlorophyll, RNA, protein (Hardwick and Woolhouse, 1967), and had declining rates of photosynthesis and respiration (Hardwick, et al, 1968). The loss in oxidative and phosphorylative activities of the mitochondria in senescing peanut cotyledons (Arachis hypogaea) was accompanied by swelling and disintegration of the structure and internal organisation of the mitochondria (Cherry, 1963). There was also a change in the effective utilization of respiratory substrates, from α - ketoglutarate to isocitrate, in senescing soybean cotyledons (Howell, 1961), which suggested the operation of the glyoxalate res-

piratory cycle. Gibbs and Beevers (1955) reported on the increasing importance of the hexose monophosphate pathway in differentiating and ageing leaf, stem and root tissues in a variety of plants. However, Baur, et al (1968) found that both respiratory pathways were operative in senescing tobacco leaves, and there was no shift towards any one particular respiratory pathway as a result of ageing.

The pattern of translocation of assimilates in young soybean trifoliate leaves (Glycine max) changes as the leaves become fully expanded (Thrower, 1962). Young leaves of less than 30% the adult size mainly imported assimilates from the lower leaves without exporting any of their assimilates. At between 30% and 50% the adult size, leaves simultaneously imported and exported their assimilates. The rate of growth of young leaves reached a maximum when about 50% the adult size was attained. At this stage, the amount of import decreased whilst the amount of export increased. The young expanding leaves were induced to absciss prematurely when darkened: these darkened leaves also had lower rates of assimilation (Thrower, 1964). Using a variety of treatments which involved the maintenance of higher levels of carbohydrates, by reducing the "loss" to other parts of the plant, the longevity of the darkened leaves was enhanced.

The movement of translocates in wheat leaves followed a very striking and precise pattern, in that each leaf (except for the first) was supported during its early development by metabolites from the lower leaves. The amount of translocate which was imported, reached a maximum during the period when the leaf attained a maximum rate of expansion, and then declined as the rate of growth declined. As the leaf ceased to grow, the level of import became very low (Doodson,

et al, 1964). Sucrose was the main form of carbon being translocated in tobacco plants (Shiroya, et al, 1961).

Chibnall (1954) showed that the rapid loss of chlorophyll and protein from the lamina of detached Phaseolus vulgaris leaves was arrested when roots were induced to form from the petioles of these leaves. There was a reduction in the rate of protein breakdown and the rooted leaves had a prolonged lifespan. Rooting of detached leaves of tomato plants also resulted in the restoration of chlorophyll and reduction of RNAase activity (McHale and Dove, 1968). Exudates from the stumps of Helianthus annuus were separated by paper chromatography and found to consist of two fractions, one of which induced active cell division in the tissue cultures of soybean callus (Kende, 1964) — a test which was specific for the detection of kinin-like substances.

The senescence of an attached bean leaf was delayed when the stem apex was removed (Das and Leopold, 1963), resulting in the green leaf having a high level of photosynthetic activity and dry weight. Direct application of kinetin to the leaf caused a delay in senescence in a manner which was similar to that of stem decapitation.

Kinetin (= 6 - furfurylaminopurine) delayed senescence of detached Xanthium leaves by maintaining a high ratio of DNA : RNA (Osborne, 1962), by reducing the activities of ribonuclease and deoxyribonuclease and restoring the RNA and DNA contents to their original levels in detached barley leaves (Srivastava and Ware, 1965). It was hypothesised that kinetin delayed senescence by suppressing the destruction of RNA, and at the same time, stimulating its production (Srivastava, 1965).

There was active accumulation of aminoacids in kinetin - treated, excised leaves of Nicotiana rustica, which resulted in protein synthesis and growth of the treated leaves (Mothes, et al, 1961). The direct application of kinetin to darkened, intact soybean leaves caused an increase in the amount of labelled assimilates in the darkened leaves, with a consequent increase in their longevity (Thrower, 1964). In the presence of ribonuclease, the accumulation of soluble substrates in kinetin - treated oat leaves was inhibited (Gunning and Barkley, 1963). Instead of causing active protein synthesis, Mizrahi, et al (1970) suggested that kinetin delayed senescence through retarding the rate of protein degradation. The presence of kinetin caused suppression of activities of peptidase in detached barley leaves (Srivastava and Arglebe, 1968) and protease in detached oat leaves (Martin and Thimann, 1972).

Albino leaves of barley do not respond to cytokinin treatment (Srivastava, 1963). Similarly, the presence of cytokinins had no effect on the total synthesis of protein and RNA in yellow Xanthium leaves which contained degrading chloroplasts (Osborne, 1967). The fact that only green tissues responded to cytokinin treatment led Richmond, et al (1971) to suggest that kinetin has a primary effect upon the hydration and permeability of the chloroplasts and their membranes, rather than having a direct effect upon the apparatus for protein synthesis.

Using both analytical and histochemical techniques, Shaw, et al (1965) demonstrated that losses in both nuclear and chloroplast RNA and total protein, preceded the rapid disintegration of chloroplasts in detached, senescing wheat leaves. The loss in DNA, hist-

one and nuclear protein were delayed until the disintegration of the nucleus. Application of kinetin delayed the onset of these degradative processes. Similarly, kinetin was shown to induce an increase in the amount of endoplasmic reticulum and ribosomes in the mesophyll cells, and delayed the degeneration of mitochondrial cristae, collapse of chloroplast grana, and abnormalities in the fine structure of the nucleus, which would otherwise have occured in senescing, detached wheat leaves (Shaw and Manocha, 1965). Another cytokinin, benzimidazole, delayed the senescence of detached wheat leaves by restoring photophosphorylation and preserving the shape, grana and intergrana lamellae, and the electron - dense particles of the chloroplasts (Waygood, 1965). Thus, cytokinins appear to maintain the membrane integrity of the cell organelles.

Cytokinins or gibberellins were shown to stimulate leaf growth of Raphanus sativus and germination of Nicotiana tabacum seeds in the dark, and it was concluded that the mode of action of cytokinins bears a close relationship to carbon metabolism (Kuraishi, et al, 1968). The presence of p-chloromercuribenzoate (PCMB), an -SH inhibitor, enhanced the cytokinin - induced physiological effects but not of those induced by gibberellins. Amytal and alloxan produced the same phenomenon as PCMB, but not the inhibitors of glycolysis, TCA cycle, cytochrome oxidase or oxidative phosphorylation.

In tobacco leaf discs, kinetin promoted nucleic acid and protein synthesis only when the endogenous level of sucrose was sufficiently high. Nicotiana rustica has a low level of endogenous sucrose, and external application of sucrose or exposure of the leaf discs to light promoted their response to kinetin (Sugiura, et al, 1962). Kinetin

and GA promoted growth and breakdown of starch in excised, young wheat coleoptiles, and also induced the release of reducing sugars from wheat endosperm tissue (Boothby and Wright, 1962). Thus, kinetin stimulated the process of starch degradation (Dennis, et al, 1967), and GA had been shown to increase the amylase activities of barley endosperm (Paleg, 1960a, 1960b, and 1961). Berridge and Ralph (1971) concluded that kinetin mobilized starch reserves and increased the flow of sugars, for metabolism.

Naphthaleneacetic acid (NAA) had little or no effect on the senescence of detached leaves of broccoli (Brassica oleracea) or Xanthium pennsylvanicum. When used in combination with kinetin, there was a synergistic uptake of aminoacids in the treated leaves, although the senescence - delaying effects of kinetin alone, was reduced by the mixture of NAA and kinetin. von Abrams and Pratt (1967) suggested that the ability of kinetin in delaying senescence was not dependent on directed transport and gross accumulation or mobilization of protein precursors. These same workers later failed to detect synchrony between changes in the levels of chlorophyll and RNA, in broccoli leaves, in the presence of actinomycin - D and kinetin or NAA, thus failing to substantiate the proposal that kinetin regulated senescence by a direct effect upon DNA - dependent RNA synthesis (von Abrams and Pratt, 1968). They suggested that the regulation of senescence may be effected by hormones upon DNA - dependent RNA synthesis.

Sacher (1967) showed that NAA caused a considerable enhancement of incorporation of labelled precursors into RNA and protein of all subcellular fractions, and induced a net synthesis of RNA and protein,

in bean endocarp tissue. Actinomycin - D inhibited the auxin - induced protein synthesis, which indicated the primary effect of NAA on the synthesis of RNA. The effect of kinetin on RNA synthesis of bean endocarp tissue was variable, and apparently unrelated to the amount of endogenous or added auxin. NAA also caused a substantial decrease in RNAase activity (Sacher, 1969).

In explants of Phaseolus vulgaris, NAA caused a two - step effect on leaf abscission (Rubinstein and Leopold, 1963). The first induction period occured when abscission was inhibited by NAA, but during the latter step, abscission was promoted by NAA. The abscission response was correlated with the time of application of auxin, rather than with the gradient of auxin present in the abscission zone, or with the total concentration of auxin that may have been present in that region. With increasing age, the bean leaves showed a decreasing response to added auxin (Chatterjee and Leopold, 1965).

The retardation of senescence by 2,4-dichlorophenoxyacetic acid (2,4-D) was effective in freshly detached autumnal leaves, but not in freshly detached young leaves, of Prunus. However, the effectiveness of 2,4-D in young leaves could be demonstrated only when the young leaves had been detached for some time, and had incurred a considerable loss of protein. The auxins 2,4-D and 2,4,5-T participate in the balance of hormonal factors which regulate protein metabolism (Osborne, 1959; Osborne and Hallaway, 1964). In senescing bean leaves the amount of bound auxin decreased as the amount of free auxin and tryptophan increased. By removing the portion of stem above the leaf under investigation, senescence was delayed and the conversion of bound to free auxin was reduced (Wheeler, 1968).

Benzyladenine (BA) induced the senescence of untreated leaves, in a manner similar to the senescence - inducing effects of stem apices, flowers and fruits (Leopold and Kawase, 1964). Thus, application of BA to certain leaves intact on the bean plant (Phaseolus vulgaris) stimulated the growth of untreated leaves but inhibited the growth of untreated leaves. When applied as a lanolin paste to axillary buds under apical suppression, BA stimulated DNA synthesis and activated axillary bud growth in tobacco plants (Schaeffer and Sharpe, 1964). When applied to the primary leaves of intact bean plants, the delayed senescence of both the leaves and the entire shoot was manifested in higher levels of chlorophyll, protein, RNA and ribonuclease at all stages of development (Fletcher, 1969). The effect of BA was independent of light intensity.

Auxins delay senescence of tissue segments by maintaining the selective permeability of membranes (Sacher, 1957, 1959). The level of auxin decreased with increasing plant age (Shoji, et al, 1951), but the level of IAA - oxidase increased with increasing age of the tissues, and also increased from apical to basal tissues on the same organ (Morgan, 1964). Monophenols which are cofactors of IAA - oxidase in vitro, accelerated abscission of cotton explants by stimulating the decarboxylation of IAA, whereas, orthodihydroxyphenols which inhibit IAA - oxidase in vitro, also inhibited the decarboxylation of IAA (Schwertner and Morgan, 1966). Results of these experiments support the hypothesis that IAA - oxidase regulates endogenous levels of IAA in a plant.

Endogenous auxins from young leaves enhance the abscission of mature leaves on the same plant (Jacobs, 1958); application of IAA

in substitution for axillary buds also caused enhanced abscission of mature leaves. External application of IAA (indolyl - 3 - acetic acid) stimulated the movement of substances towards the point of application, possibly resulting from the direct stimulation upon growth (Booth, et al, 1962).

In his survey, Jacobs (1962) proposed a system of "auxin - auxin" balance for the control of abscission of leaves, flowers and fruits. Thus, the position of application of auxin on the explant determined whether abscission was accelerated or inhibited (Abeles, 1967). Distal application of auxin inhibited abscission because of the rapid translocation of auxin to the abscission zone, whereas, proximally applied auxin stimulated abscission because of its inability to be transported rapidly to the abscission zone, and because of the dominating effect of continuous evolution of endogenous ethylene. The effect of kinetin on the acceleration or retardation of abscission in Phaseolus explants was also dependent on the position of application of kinetin (Osborne and Moss, 1963). Kinetin applied directly to the abscission zone retarded senescence of the cells comprising the separation layer and hence retarded abscission. When applied to the petiolar or pulvinar ends of the explants, abscission was accelerated. It was suggested that the effect of kinetin in mobilizing metabolites to the treated areas, affected the onset of abscission, and the experiments also suggested that separation is dependent on the senescence of cells comprising the separation layer in the abscission zone.

Deciduous trees which senesce in the autumn respond to gibberellic acid (GA) treatment by delaying chlorosis and abscission of the

foliage and showing renewed shoot growth (Brian, et al, 1959). GA completely stopped the net degradation of chlorophyll and protein in Rumex leaf discs, and was fully effective even when applied to leaf discs undergoing the middle of the log - phase of chlorophyll loss (Goldthwaite and Laetsch, 1968). These results were similarly obtained when kinetin or 6 - benzylaminopurine were used. The addition of GA to leaf discs of dandelion (Taraxacum officinale) retarded their senescence and delayed the decline in levels of chlorophyll, protein and RNA (Fletcher and Osborne, 1966); using actinomycin - D, it was shown that the effect of GA in retarding leaf senescence was mediated through a regulation of DNA - dependent RNA synthesis. Senescence of the shoot apex of "Alaska" peas was correlated with reduced levels of endogenous gibberellin, RNA and protein (Ecklund and Moore, 1968). There was a similar reduction in the level of gibberellin during the senescence of nasturtium leaves, Tropaeolum majus, together with an increase in the concentration of abscisin (Chin and Beevers, 1970).

Osborne (1955) suggested that leaf abscission may be controlled not only by endogenous auxins but also by substance(s) produced as the leaf matured, these substance(s) reached a maximum level at senescence. She found that diffusates from the cut petioles of senescent leaves of several plant species were able to accelerate abscission of Phaseolus explants. Aqueous extracts from senescent leaves of Corchorus olitorius applied to the leaf axils also accelerated the formation of the separation layer (Sen, 1968). The activity of mitochondrial succinoxidase of Ricinus communis endosperm was inhibited by aqueous extracts of senescent leaves of Ricinus communis, Spinacea

oleracea and Cucumis sativus (Baddeley and Simon, 1969). The inhibitory effects of these aqueous extracts became more severe as senescence progressed, and they were thermostable and dialysable. In each of these species, the free fatty acid content of mature green tissues was negligible but increased in concentration as senescence progressed. Pure fatty acids like oleic, linoleic and linolenic were found to be equally inhibitory to mitochondrial succinoxidase. It was suggested that the inhibitory properties of the aqueous extracts from senescent leaves were attributed to their free fatty acids. The rise in free fatty acid content in senescent cotyledons of Cucumis sativus resulted from a loss of chloroplast free fatty acids (Draper and Simon, 1971).

Abscisin, a plant hormone isolated from cotton burs (Liu and Carns, 1961) and from various organs of a variety of plants (Cornforth, et al, 1966) is an accelerant of abscission. It enhanced the senescence of first seedling leaves of barley by causing an accelerated loss of chlorophyll and an increase in chromatin - associated ribonuclease and deoxyribonuclease activities (Srivastava, 1968); kinetin completely reversed the senescence inducing effects of abscisin.

When debladed plants of Coleus blumei were placed in a horizontal position, the amount of ethylene production and the rate of petiole abscission were greater than in control plants placed in a vertical position (Abeles and Gahagan, 1968). The increased rate of ethylene production was thought to be the cause of the increased rate of abscission. In a separate publication, the same authors, Abeles and Gahagan (1968b) reported on the accelerated abscission of explants of Coleus blumei, Gossypium hirsutum and Cassia fistula on application

of ethylene, a gaseous hormone. Ethylene stimulated active synthesis of RNA and protein in the abscission zone of petioles and enhanced their abscission (Abeles and Holm, 1966, 1967; Holm and Abeles, 1967). The effect of actinomycin - D on ethylene treatment indicated that ethylene regulated abscission through the control of specific RNA's: actinomycin - D inhibited ethylene - stimulated abscission and inhibited the incorporation of P^{32} into RNA. Ethylene hastened the dissolution of cells in the abscission zone without stimulating degradative processes (Abeles, et al, 1967), and the abscission response to ethylene occured only when tissues have begun senescence (Abeles, 1967; de la Fluente and Leopold, 1968). The dissolution of pectic substances between cells was brought about by an increasing pectinase activity which was not present in freshly harvested explants of Phaseolus vulgaris (Morre, 1968), but was present in senescent tissues.

Contrary to the findings of Abeles and his associates, Valdovinos and Muir (1965) reported that the interference of protein synthesis by application of several D-aminoacids to the debladed petioles of Gossypium hirsutum, promoted their abscission. Chloramphenicol, actinomycin - D, ethionine and azaguanine, which are inhibitors of protein synthesis, also promoted abscission of debladed petioles of Coleus blumei, Gossypium hirsutum, Impatiens sultani and Nicotiana tabacum (Valdovinos, et al, 1967), and it was suggested that abscission occured as a result of reduced rates of protein synthesis. Abeles (1967) also found that some of the D-aminoacids were more effective stimulators of abscission than L-aminoacids, but the D-aminoacids were also better stimulators of endogenous ethylene production than L-aminoacids; enhanced abscission was attributed to the greater production

of endogenous ethylene.

Anatomical changes which precede the abscission of bean petioles (Phaseolus vulgaris) have been described by Brown and Addicott (1950). Evidence was presented which suggested the effect of water stress in bringing about abscission. The xylem vessels were plugged with tylose, and callose disappeared from the phloem, in the abscission zone of bean explants prior to their abscission. Application of ethylene or NAA (at 10^{-5}M) accelerated abscission, by causing an increase in the amount of tylose plugged vessels and reducing the amount of callose in the seive tubes (Scott, et al, 1967). At a concentration of 5×10^{-4}M, NAA inhibited abscission by reducing the amount of tylose plugged vessels and increasing the amount of callose in the seive tubes. Tylosis of xylem causes water stress in the tissues distal to the separation zone, and clearing of the seive tube callose facilitates translocation of metabolites from the distal to the proximal tissues. Scott and Leopold (1966) found that nutrients were mobilized out of the distal cells, during senescence and development of abscission.

Abscisin and GA are accelerants of abscission in Gossypium hirsutum explants, but they exert different effects on the abscission zone (Bornman, et al, 1967). Abscisin caused lysigenous breakdown of parenchyma cells in and often distal to the abscission zone, multiple fragmentation of cell walls, restricted cell division and formation of a well defined separation layer, restricted tylosis of xylem vessels distal and proximal to the abscission zone, and caused petiole abscission within 24 hours of treatment. GA accelerated abscission by inducing cell division and the formation of a separation layer,

caused schizogenous breakdown of the middle lamellae of anti- and periclinal walls, tylosis in the xylem vessels, partial obliteration of procambium in the region of the abscission zone, and caused separation to take place within 48 hours of treatment. IAA retarded abscission by causing petiolar growth, cell division in the abscission zone and therefore the formation of a discrete separation layer, prolific formation of tylose in the xylem vessels and caused separation to take place 120 hours after treatment. Bornman, et al (1967) suggested that the plugging of xylem vessels with tylose was of secondary importance and was not causal to the dislodging of petioles; its formation was more directly correlated with excission and with ageing.

General summary

The senescence process which occurs in the lamina of a variety of plants is generally characterised by the loss of chlorophyll, fresh weight, RNA, protein, starch, nitrate reductase activity and reduced rates of photosynthesis and respiration; and accompanied by a rise in activities of peroxidase, glucose - 6 - phosphate dehydrogenase, RNAase, peptidase, pectinase, IAA - oxidase, and level of free fatty acids; together with the destruction of the fine structure of chloroplasts, nuclei and mitochondria. With increasing age of an organ, the level of endogenous auxin and gibberellin gradually falls, whilst the level of abscisin and ethylene production increases. It appears that the physiological state of an organ is determined by the balance of natural hormones.

Senescence of an attached leaf is delayed upon removal of the

stem apex, flowers or fruits. Detached leaves delay their senescence when rooted, or when supplied with cytokinins, auxins or gibberellins. These treatments bring about a reversal of the biochemical processes which are known to occur during senescence, by maintaining the membrane integrity and intrinsic functions of cell organelles, and preserving the apparatus for nucleic acid and protein synthesis. The consequent resumption of growth, increases the longevity of the leaves.

Senescence generally leads to abscission of the whole organ. Auxins and cytokinins retard senescence and abscission, but gibberellins and abscisin enhance abscission by exerting different effects in the abscission zone. Plant organs become susceptible to ethylene only when they are in a senescent state, and ethylene production increases with increasing age of the plant.

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EXPERIMENTAL WORK

Physiological and biochemical effects of Sirex mucus on the foliage of Pinus radiata

Introduction

Studies on the tree - insect interaction so far, have suggested that healthy vigorous trees are better able to resist a Sirex attack than weak or damaged trees, through their increased output of resin and polyphenols (Titze and Mucha, 1965; Coutts and Dolezal, 1966b). The insect and fungus are dependent on the tree as a means for the production of the future generation, and therefore try to create a favourable environment for this purpose, by causing the host to weaken and starve through premature yellowing and abscission of foliage. Coutts (1969) demonstrated that the mucus secretion which is deposited into oviposition tunnels by the female wasps, is the main factor in causing the premature yellowing and abscission of foliage, but not death of the tree. The physiological activity of Sirex mucus is thermostable.

The fungus causes desiccation of the wood and death of ray parenchyma cells, and there is no evidence to suggest that fungal hyphae occlude the xylem tracheids (King, 1966). Moreover, partially purified fungal culture filtrates only wilted young P.radiata saplings with intact roots, without inducing chlorosis (Titze, 1970).

Inoculations of aqueous solutions of either mucus or fungal suspensions into P.radiata gave rise to symptoms of a "light Sirex attack", but the use of a mixture of mucus and fungus together resulted in the development of symptoms of a "heavy Sirex attack" and con-

sequent death of the tree (Coutts, 1969b).

In the following experiments, measurements are made of the levels of physiological and biochemical changes in the foliage of P.radiata which show obvious susceptibility to Sirex mucus. A comparison is made of these changes in the foliage of both intact and excised branches. Experiments of this nature are invariably subjected to seasonal fluctuations, and whilst it is not practicable to compare absolute values for each of the items under consideration, for any two series of experiments conducted at different times of the year, one may be able to observe definite trends in their responses to treatment.

Methods

a. Treatment

i) Whole tree experiments

Vigorous rammets of P.radiata, 5 - 6 years old, were chosen from cloned material grown at the* Forest Research Institute Field Station at Hobart. The selected trees had similar appearances and grew within close proximity of one another. A sample consisting of four trees, each received fresh mucus solution (0.5g/10ml) or **autoclaved mucus solution (0.5g/10ml), and the control sample received deionised water (10ml). Test solutions were introduced into radial bore holes, 3mm (diameter) X 2cm (depth), spaced at 2.5cm intervals, over a surface area which covered 30cm of the circumference

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* The Forest Research Institute has, since 1/7/75, become part of the C.S.I.R.O., and is now known as C.S.I.R.O., Division of Forest Research.

** Mucus solution was autoclaved for 15min, at 1 atmosphere pressure.

of the tree trunk, between the two lower whorls of branches. The effects of treatment were investigated in 50 1-yr old fascicles taken from branches of the third and fourth whorls behind the shoot apex. Due to the irregular appearance of effects along the length of the needles, with a broad tendency for a progression from apex to base, investigations were conducted on a standard 1-cm segment taken 4cm from the base of the fascicle.

ii) Experiments on excised twigs

Branch tips about 50cm in length, were selected from trees which had previously been shown to have high susceptibilty to mucus. Each sample consisted of six twigs which were stood in test solutions contained in glass conical flasks, and maintained in the phytotron of the Botany Department where daily temperatures were 20 ± 3°C, throughout the experiment. The test solutions consisted of fresh mucus (1%, 10ml), autoclaved mucus (1%, 10ml) and deionised water (10ml); and when depleted, were replenished with deionised water. In order to prevent excessive accumulation of resin exudates on the surfaces of the cut stems, fresh cuts were made on the stem ends on alternate days. Investigations were also concentrated on standard 1-cm segments of 50 1-yr old fascicles.

b. Preparation of standard 1-cm needle segments

Needle fascicles were trimmed to 5cm lengths (from their bases), and the cut ends placed over a section cutter which consisted of two mounted razor blades spaced 1cm apart. Segments were cut by means of pressing the fascicles against the razor blades with a block of wax. The segments thus obtained were placed in moistened, filter paper lined petri dishes, over a tray of ice, and used as soon as possible.

The remainder of the fascicles were stored in the freezer for further use.

c. Analysis

i) Weight

The fresh weight of each sample of 50 needle segments was noted, and the samples placed in a 100°C oven for 20hr. They were transferred to a desiccator containing silica gel, to cool, and the dry weight then determined.

ii) Respiration

Duplicate samples of 50 needle segments were weighed, and their respiratory rates in darkness were determined in a Warburg apparatus at 25°C. Details of this procedure are given in the appendix. These same needle segments were then used for further chemical assays.

iii) Cold water extracts

Extracts were obtained by grinding 50 needle segments in a pre-chilled mortar and pestle, with sand and 10ml of deionised water, at 2°C, and centrifuging the slurry at 20,000g for 10min. The supernatant collected was concentrated by dialysis against a saturated solution of polyethylene glycol (MW 4,000) to a final volume of 0.5ml. In subsequent experiments with twigs, this latter procedure was eliminated. As a result of the different volumes of extracts from tree and twig experiments, it was necessary to use different proportions of extract solutions and reagents in all of the following procedures.

1. Soluble protein content was measured by the method of Lowry, et al (1951) and by the Biuret reaction of Layne (1957).
2. Peroxidase was assayed spectrophotometrically by the procedure of Maehly and Chance (1955), with o-dianisidine as substrate

(1.25×10^{-3}M, in 0.1M acetate buffer, pH 4.5). The course of reaction was recorded on a Hitachi chart recorder attached to the spectrophotometer.

3. Amylase was assayed by the method of Bernfield (1955), in a solution of phosphate buffer, 0.1M, pH 6.9.

4. Electrophoresis on 8% acrylamide - starch gel was carried out according to the procedures of Mills and Crowden (1968). Each slab of gel was sliced into four, and stained for protein (with amido black), amylase (with I_2/KI), peroxidase (with o-dianisidine and H_2O_2) and esterase (with α-naphthylacetate and Fast Blue BB).

iv) Residues from cold water extraction

After having extracted the cold-water-soluble material from tissue homogenates, the residue was used for further assays.

1. Chlorophyll was extracted from the residue with three aliquots of hot 80% ethanol, to a total volume of 10ml. The chlorophyll solution collected by centrifugation, was measured spectrophotometrically at 645nm and 663nm. The formula of Arnon (1949) was adopted for calculations of levels of chlorophylls a and b. In twig experiments, the overall chlorophyll content was measured at 665nm.

2. "Insoluble" protein from the tissue homogenates was released by hydrolysis with 0.3N KOH for 16hr at 37°C, after prior treatments with trichloracetic acid and ethanol (details are given in the appendix). The alkaline supernatant was used for protein determination by the Biuret method.

3. RNA in solution was determined from the alkaline extracts of

tissue homogenates. These extracts were prepared as in (2) above, and then adjusted to pH 2 with perchloric acid. Following centrifugation, the clear supernatant was read at 260nm in a spectrophotometer.

v) Hot water extracts

50 needle segments were similarly homogenised in a mortar and pestle with 10ml of deionised water, boiled for 2hr, cooled, and centrifuged at 1000g for 10min. The supernatant was then stirred for 1hr with 1g each of pre-swollen Zeokarb - 225 (Ac^-) and Dowex 50 (H^+). Assays made on these extracts include the following:

1. Sugar content was determined with the anthrone reagent (Trevalyan and Harrison, 1952).
2. The sugar complement was examined by thin layer chromatography on cellulose (Whatman CC 41) with solvents n-butanol : acetic acid : water (4 : 1 : 5, v/v) (upper phase), and n-butanol : benzene : pyridine : water (5 : 1 : 3 : 3, v/v). Authentic sugar samples were used as references for each chromatogram. Vizualisation was achieved with aniline hydrogen phthalate spray.

vi) Anatomy of needles

1. Structure

Transverse and longitudinal sections, 22μm thick, were cut on a freezing microtome from needles taken 0.5cm below the standard sample segments. Examination of tissues was made on sections which were unstained, stained with aniline sulphate (a stain for lignin), and stained with tannic acid - ferric chloride - lacmoid (Cheadle, et al, 1953). Photomicrographs of some of the sections were recorded.

2. Starch count

Transverse sections, 22μm thick, were cut on a freezing microtome from samples each consisting of 20 needles. These sections were stained in I_2/KI for 5min, mounted in glycerine, and viewed at 600 magnifications. The number of starch grains was counted in four separate fields of view (refer fig. 3), and an average number of grains in the four fields of view was calculated and assumed to be the total number of starch grains for that section.

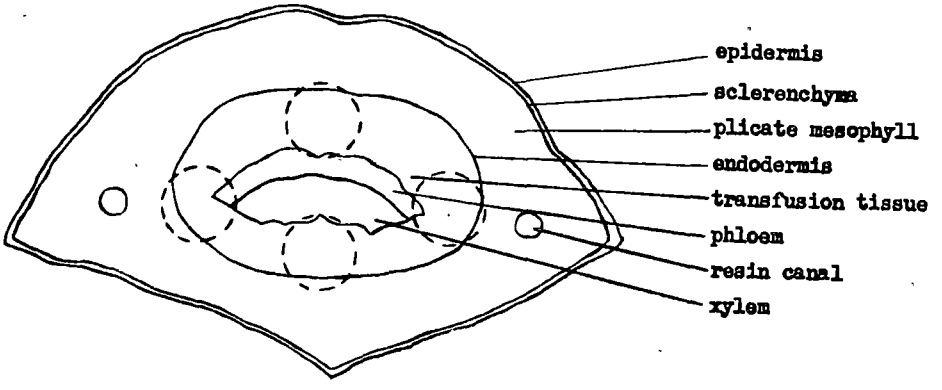


Fig. 3. Diagrammatic representation of cross section of pine needle, showing areas (enclosed in broken circles) where starch counts were taken.

vii) Statistical analysis

Sets of data obtained over specified time intervals, before and after treatment, were analysed by the t - test. The values of P are shown in legends appropriate to the figures.

Results

Morphology

Tree experiments have shown that the oldest needles turned yellow and abscissed before the younger needles in a progressive sequence; furthermore, branches lower down on the tree showed symptoms before those which were closer to the main apex. However, as trees were injected in the region of main stem between the two lowest whorls of branches, those branches immediately above the region of injection expressed very early symptoms whereas, those branches below the region of injection remained visually healthy until most of the tree had turned yellow and dry. The apical buds of all the branches wilted and turned brown about three months after treatment. By this time, the trees had become almost denuded.

The shoot apices of excised twigs also delayed the mucus - induced senescence of needles in their vicinity. Fig. 4c shows a mucus treated twig with brown - dry needles gradating to yellowish - green needles, from the lower to the upper part of the twig. Moreover, treatment with kinetin delayed the yellowing of excised twigs which were maintained on deionised water for six weeks (figs. 4a and 4b). The apices of all these branches wilted after the rest of the twig needles had died.

Similar symptoms were expressed by both trees and twigs, although the time taken for development of symptoms was not necessarily the same in both cases, partly due to variations in seasons and ambient temperatures, and the forms of the experimental material. Thus, yellowing and uniform drying of needles proceeded from tip to base, but the process of yellowing was often patchy along the length of the

(a) (b) (c)

Fig. 4. Photographs of P.radiata twigs, six weeks after excision.

(a) Control, in deionised water only.

(b) Treated with kinetin (10mg/ml), in the first two weeks of excision.

(c) Treated with Sirex mucus (1mg/ml), in the first two weeks of excision.

Fig. 5. Two suites of needles from a mucus - treated tree, 7 days after treatment. The proximal parts of needles are green and turgid, but the distal parts are chlorotic and dehydrated. Distinguishing marks on these needles are indicated.

a = "black band"
b = grey - green
c = "water mark"
d = brown - green
e = brown
f = green
g = prominent ribs

needle. As a result, formation of chlorophyll islands was common especially in the middle stages of yellowing. These chlorophyll islands either turned yellow or persisted, in which case severe loss of water from the tissues often changed their colour to a grey - green.

Peculiar to all experiments with cloned material in the period 1969 - 1972, was the appearance of "black bands" on the affected parts of needles. These "black bands" were actually translucent when viewed in transmitted light. Grey "water mark" areas also developed on the affected needles. These symptoms were expressed in the distal parts of the needles, while the proximal parts remained green and turgid when the photograph was taken one week after treatment (fig. 5).

Anatomy

Localised variations in degrees of response to mucus treatment were detected in transverse and longitudinal sections of any one sample of needles. Some anatomical changes were detected (in the segment selected for anatomical studies) two days after treatment with autoclaved mucus and about four days after treatment with fresh mucus. The early changes include loss of granules from the mesophyll, shrinking of the resin canal epithelium, and flattening of the phloem cells (compare figs. 6 and 8).

In addition to making anatomical observations on the segment specified in "Methods", anatomical observations were also made on the different regions of a day 7 mucus treated needle, as is illustrated in fig. 5. Where the needle was visually green and turgid, the tissues appeared to be healthy and without signs of disarray (fig. 10b). The yellow, grey - green, brown - green, "water mark" and "black band" regions of the treated needle showed varying degrees of dehydration

in the unlignified tissues (figs. 9 to 13, inclusive). The unlignified tissues which were affected, included the mesophyll, epithelium of the resin canals, endodermis, ray parenchyma, phloem, and pith parenchyma. Although the epidermis is unlignified, it is "protected" on its outer surface by the thick waxy cuticle, and by the sclerenchyma immediately below it, and therefore showed no apparent signs of dehydration. The transfusion tissue is only slightly lignified, but nevertheless, showed effects of dehydration.

Cells which suffered excessive water loss became flaccid, distorted in shape, and in extreme cases, completely necrosed. There appeared to be a predominance of opened stomata in sections which contained flaccid tissues, thus suggesting that an increased rate of transpiration may be the cause of water loss from the tissues. The mesophyll appeared to be most vulnerable to dehydration.

In tissues where dehydration was insignificant, the unlignified tissues and the transfusion tissue remained intact, but the mesophyll became less granular or empty, and the phloem compressed or necrosed (figs. 8, 9, 11, 18 and 19). These latter effects, i.e., chlorosis due to loss of chloroplasts from the mesophyll and phloem necrosis, are two of the primary symptoms of susceptibility to Sirex mucus. Excessive loss of water through the abnormally opened stomata is also another primary symptom, but tissue dehydration may be prevented by the presence of the fascicle sheath which is closely wrapped around a centimeter of the base of the needles. Thus, tissues from the ensheathed region of needles, after 28 or 31 days of mucus treatment, remained turgid even though the mesophyll was empty and the phloem completely necrosed (figs. 18 and 19). As the section in figure 19

Fig. 6. Transverse section of a normal, untreated needle.

a: whole section.

b: intact granular mesophyll (m), with resin canal (c) consisting of turgid epithelial cells. The endodermis (e) and transfusion tissue (t) are also turgid.

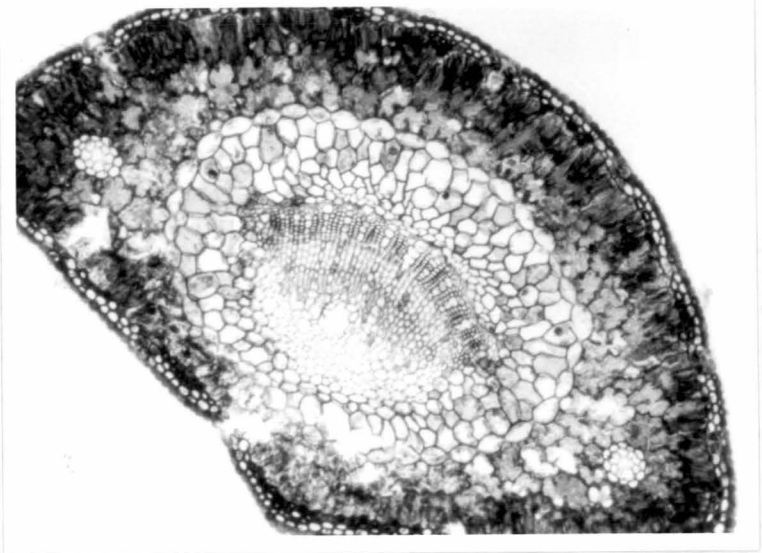
c: vascular bundle consists of regular rows of phloem (p) and xylem (x), and vascular cambium (a). The ray parenchyma (r) extends the length of the vascular bundle.

Fig. 7. Longitudinal section of a normal, untreated needle.

a: mesophyll in the normal granular state, with turgid endodermis and transfusion tissue.

b: some of the transfusion and endodermal cells are filled with starch grains (st). Ray parenchyma cells associated with the phloem often contain rhomboidal shaped crystals (cr). Phloem cells are faintly visible in the photomicrograph. Metaxylem (mx) are recognised by the presence of bordered pits on their cell walls, and protoxylem (px) by the presence of spiral lignification.

Fig. 6



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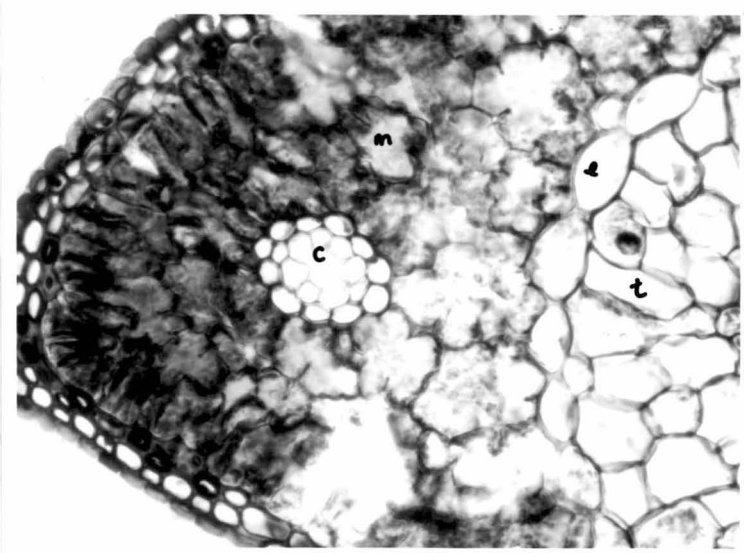

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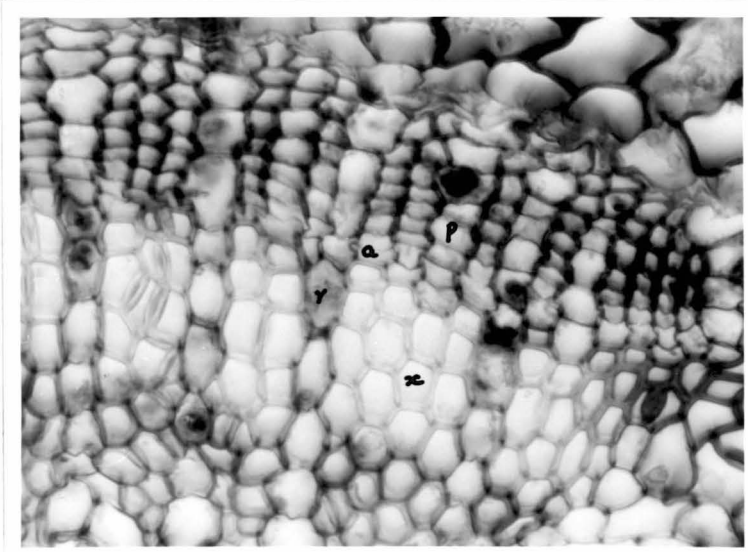

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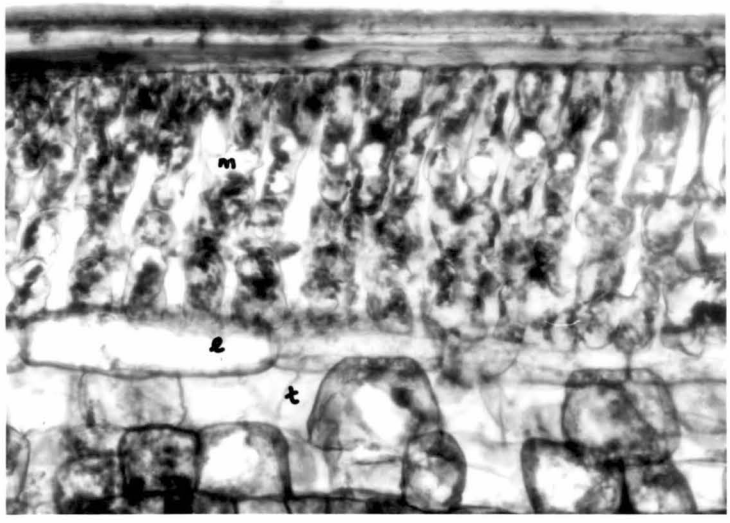

Fig. 7

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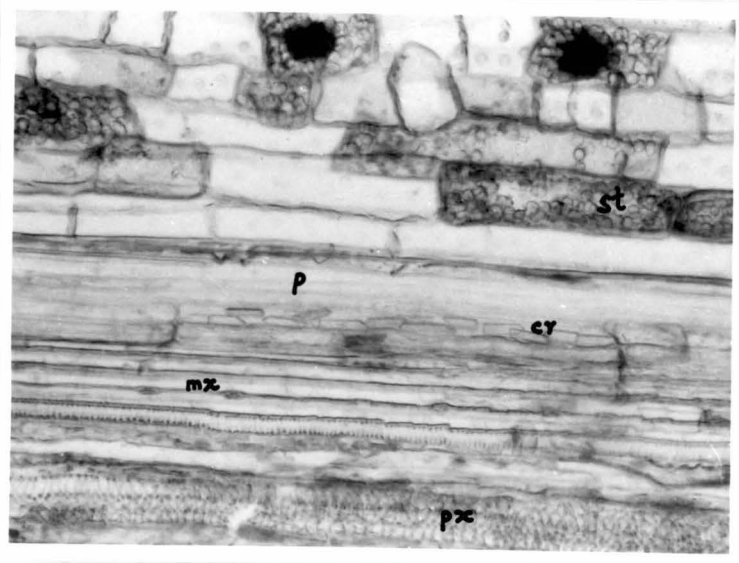

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Fig. 8. Transverse section of needle, after treatment with autoclaved mucus for two days.

a: whole section, with patches of mesophyll being torn away during sectioning.

b: mesophyll cells are turgid but less granular than normal. Epithelial cells of the resin canal are shrunken and flattened.

c: although the endodermis, transfusion tissue and xylem appear to be normal, the phloem cells are compressed. Breakdown of parenchyma (pa) in the region of the protoxylem (px), results in the formation of large holes.

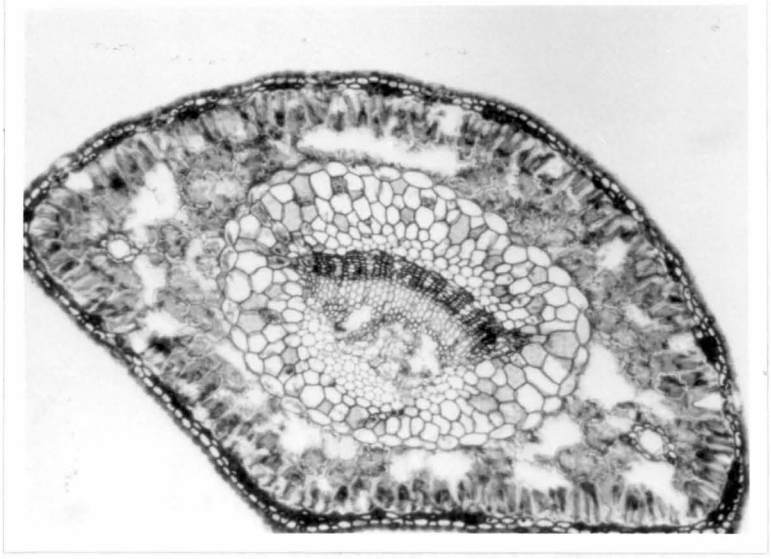
Fig. 9. Transverse section of a day 7 mucus treated needle, from the yellow, slightly dry region.

a: whole section appears to be slightly shrunken.

b: mesophyll cells, especially those adjacent to the subepidermal sclerenchyma, are flaccid and empty. The inner epithelium of the resin canal is much enlarged, and almost occludes the lumen.

c: the phloem and transfusion tissues are slightly irregular in shape. A large proportion of parenchyma at the tips of the vascular bundles have broken down.

Fig. 8



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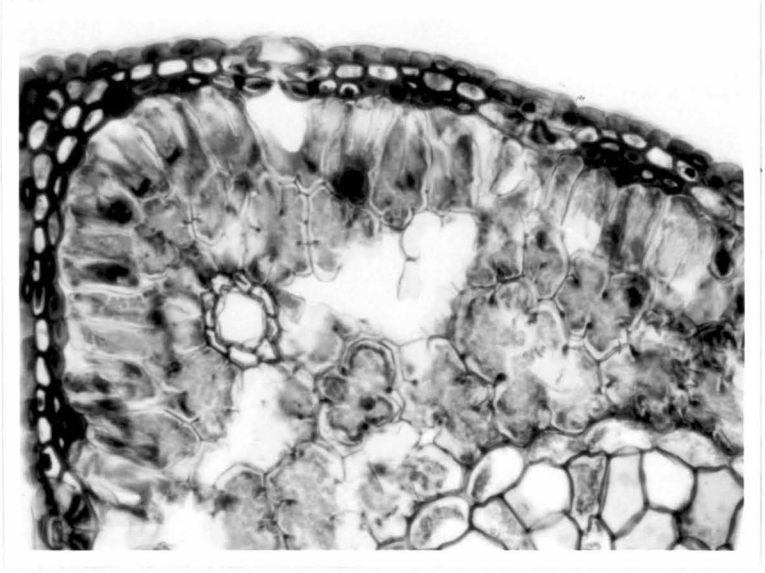

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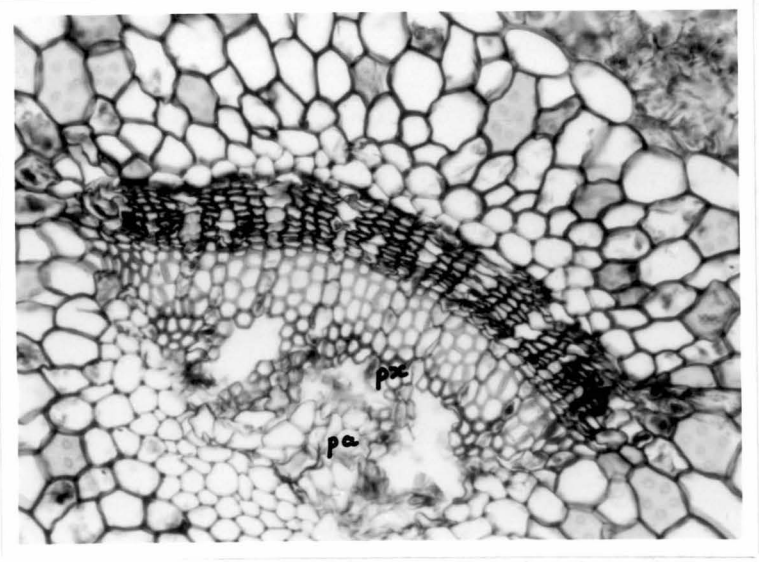

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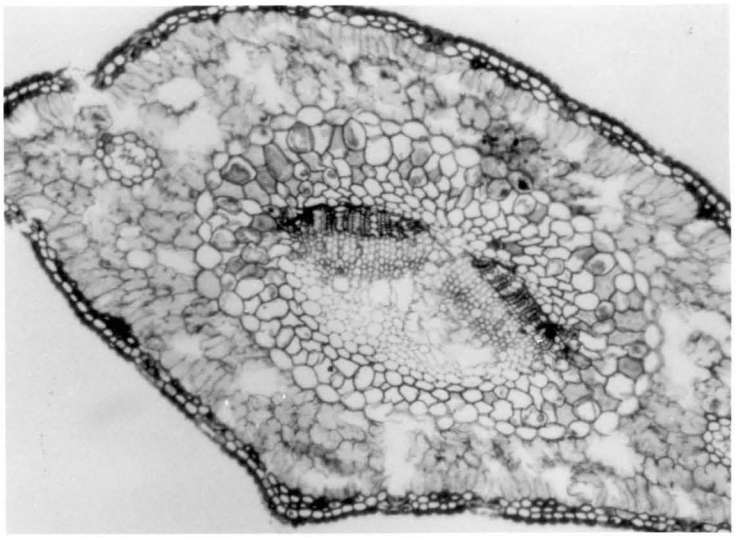

Fig. 9

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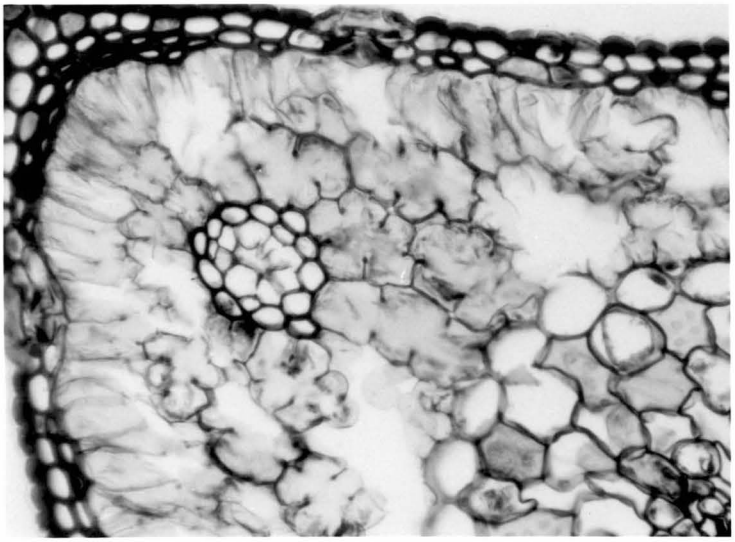

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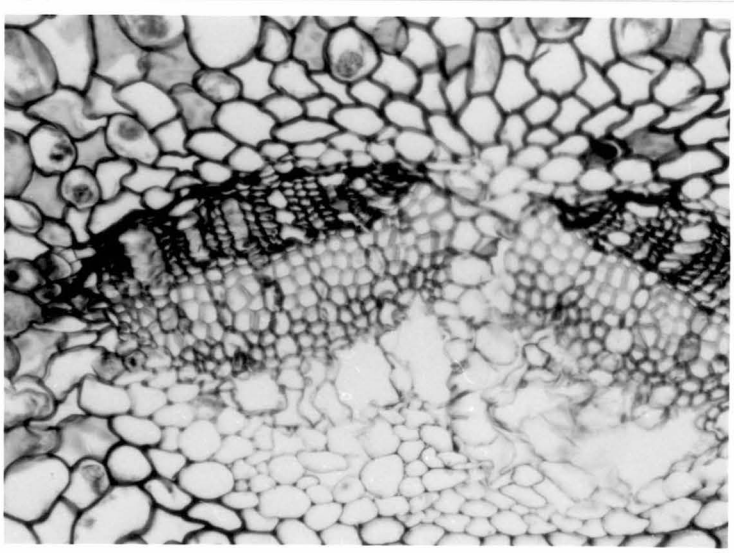

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Fig. 10. Transverse sections of a day 7 mucus treated needle.

a: section taken from the green, healthy region. All of the tissues appear normal and turgid, and the mesophyll is granular.

b: section taken from the dry, grey - green region. The needle had obviously shrunken through excessive water loss, and all of the tissues except for those which are highly lignified (i.e., sclerenchyma and xylem) or "protected" (i.e., epidermis) show effects of dehydration. Much of the parenchyma at the tips of the vascular bundles have broken down.

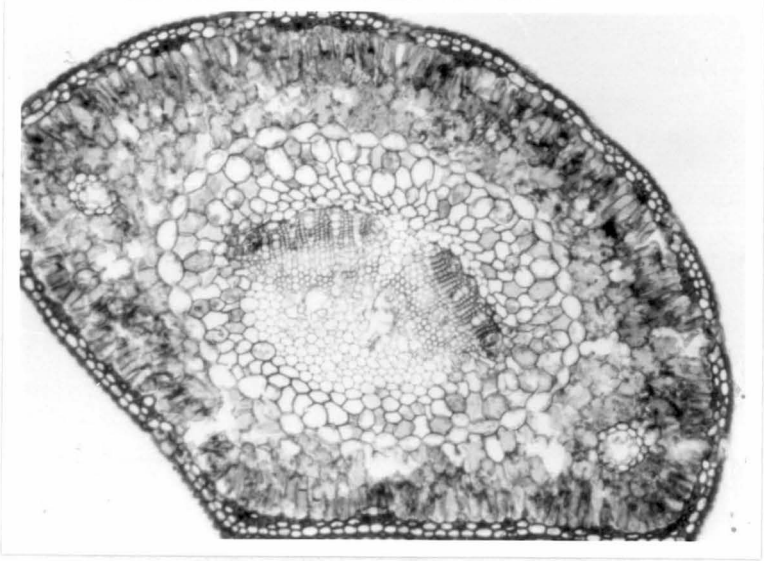
Fig. 11. Transverse section of a day 7 mucus treated needle, from the brown - green region.

a: whole section, where effects of water loss are not apparent.

b: mesophyll although intact, is almost devoid of granules. The inner epithelium of the resin canal is markedly reduced.

c: the transfusion tissue is slightly irregular, and the phloem cells appear to be slightly flattened.

Fig. 10



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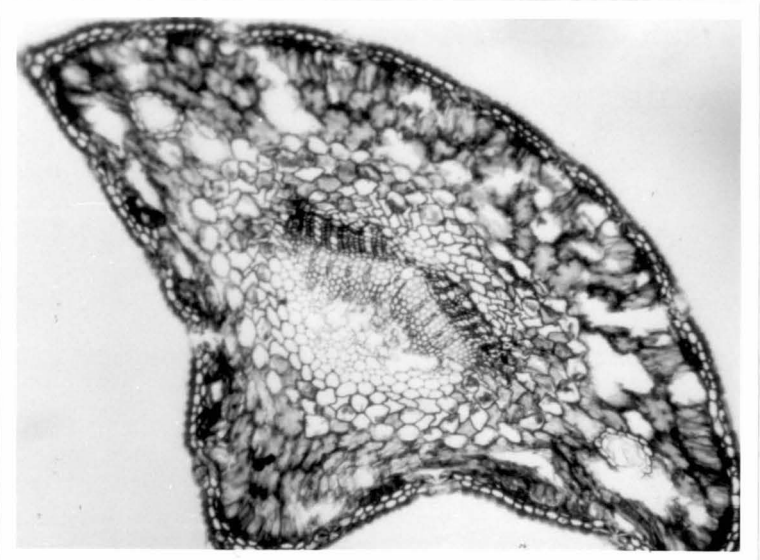

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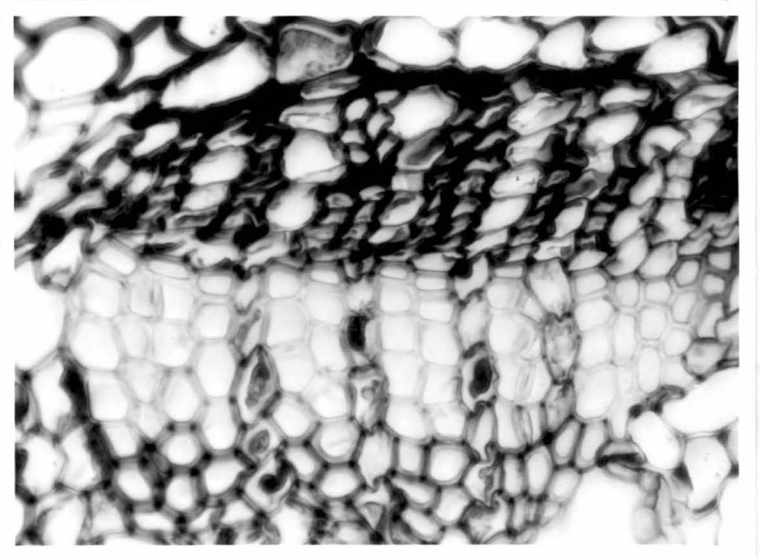

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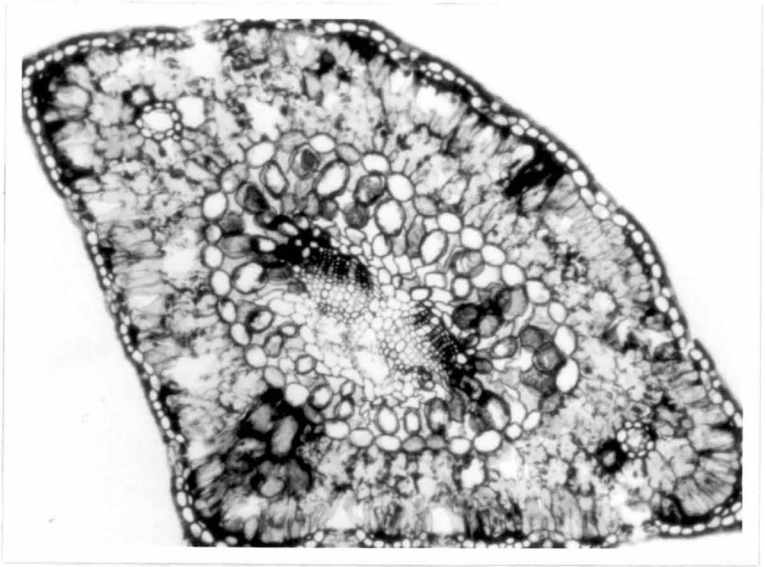
Fig. 11



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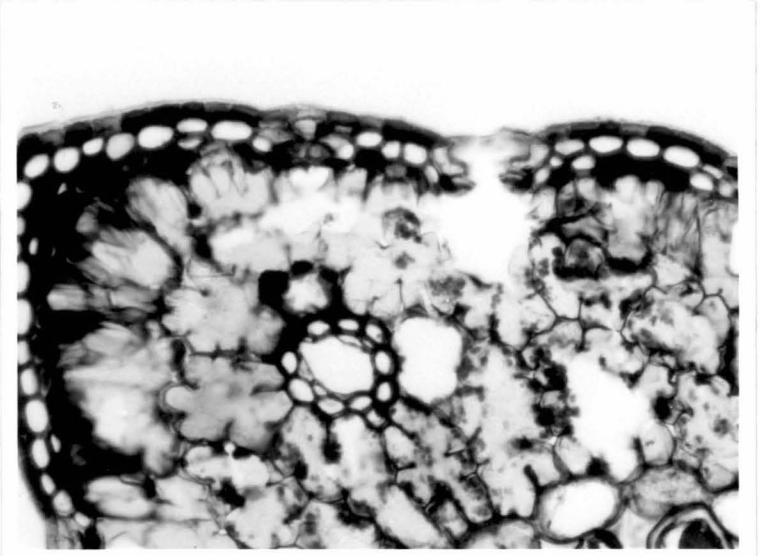

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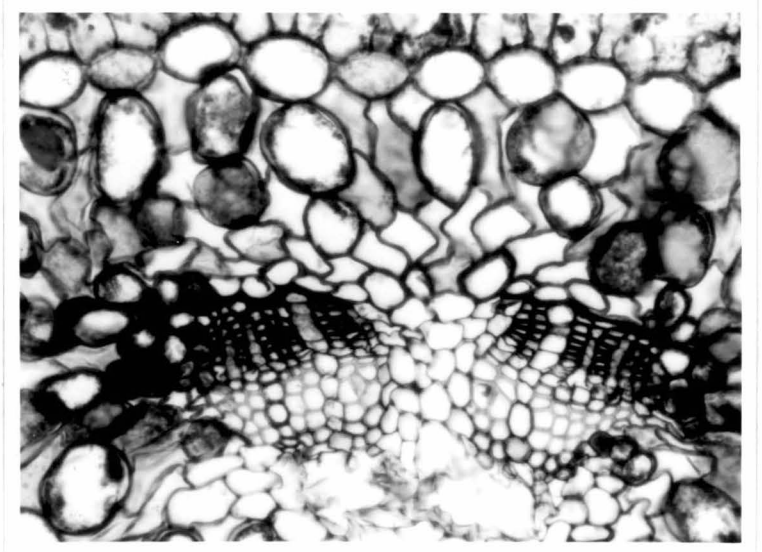

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Fig. 12. Transverse section of a day 7 mucus treated needle, from the "water mark" region.

a: whole section, shrunken through water loss.

b: mesophyll cells are breaking down, and are almost empty. The endodermis and transfusion tissue also show effects of water loss.

c: phloem cells have either become distorted in shape, or have collapsed. Ray parenchyma cells are also distorted, but some of the phloem rays have assumed a square shape through being filled with rhomboidal shaped crystals. Although the rigid, highly lignified xylem cells do not show any change in cell shape; it appears that some of the adjacent cells have separated, thereby leaving intercellular spaces (as indicated by the arrows).

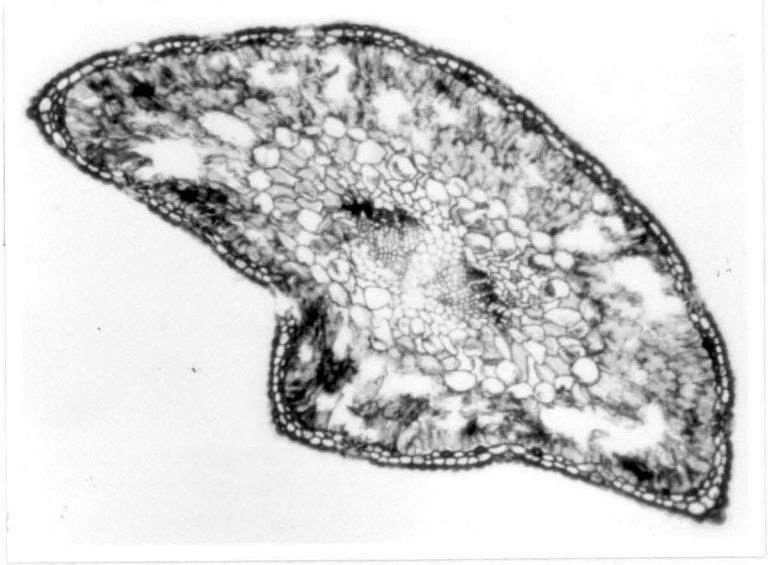
Fig. 13. Transverse section of a day 7 mucus treated needle, from the "black band" region.

a: whole section, showing effects of severe water loss.

b: the mesophyll, endodermis and transfusion tissue are flaccid and much distorted in appearance. The resin canal is stretched, and the epithelial cells have collapsed.

c: phloem and ray parenchyma cells are in the process of collapsing. Crystals are clearly visible in the phloem rays.

Fig. 12



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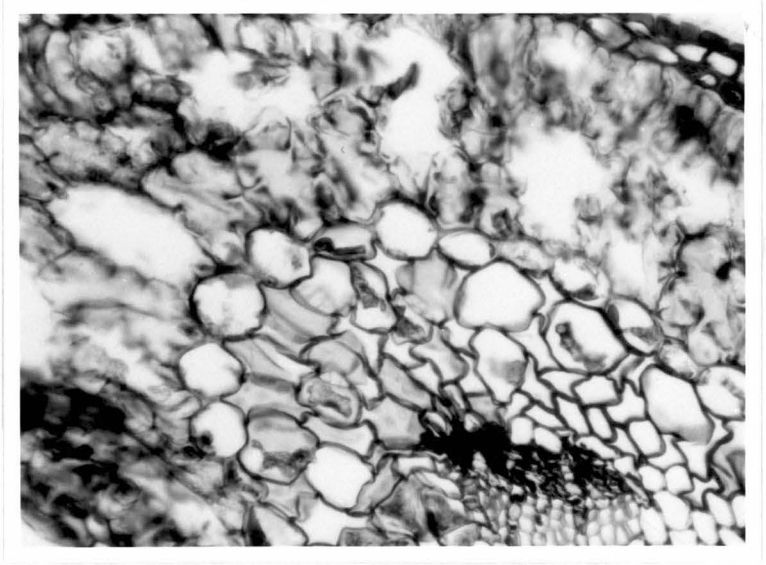
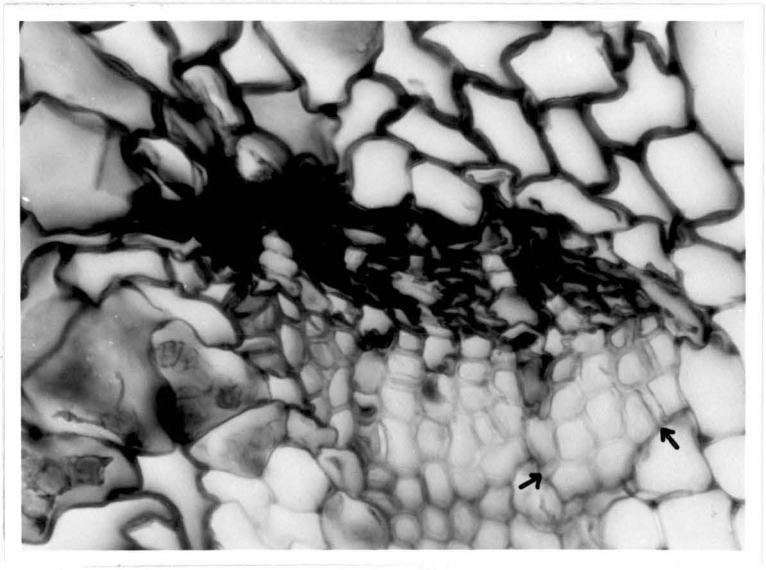


b

(x 250)



c

(x 625)

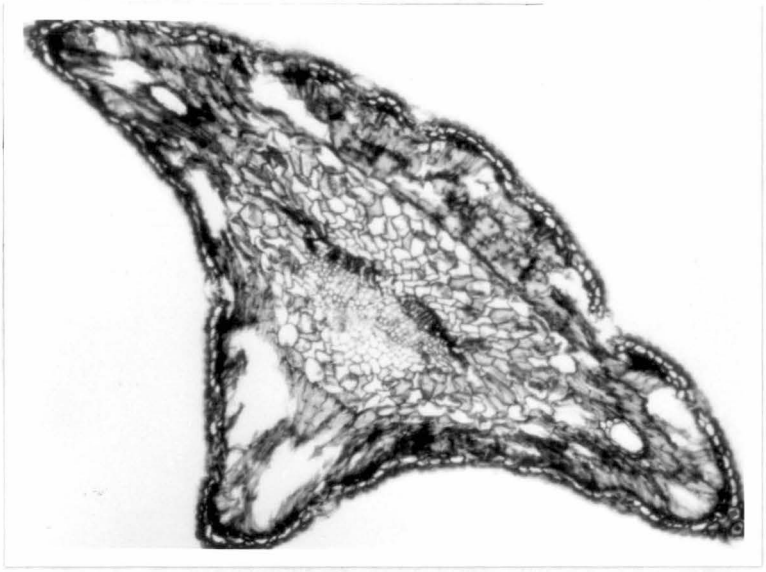
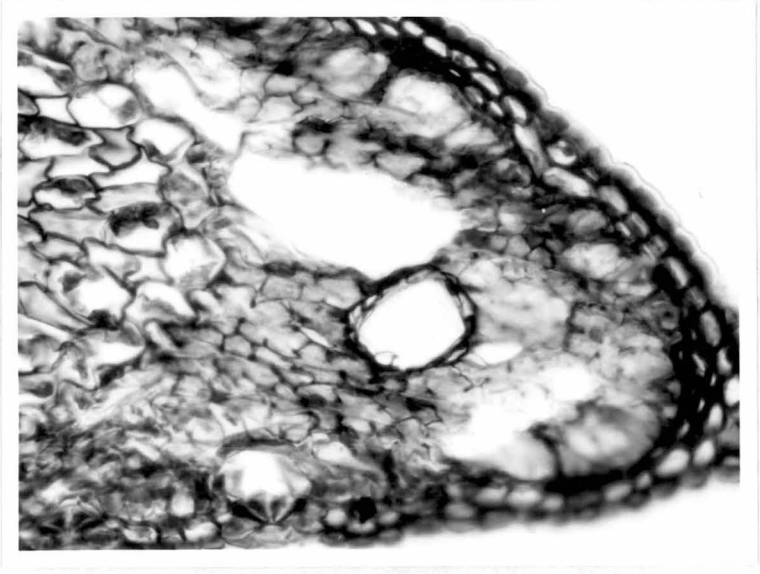


Fig. 13

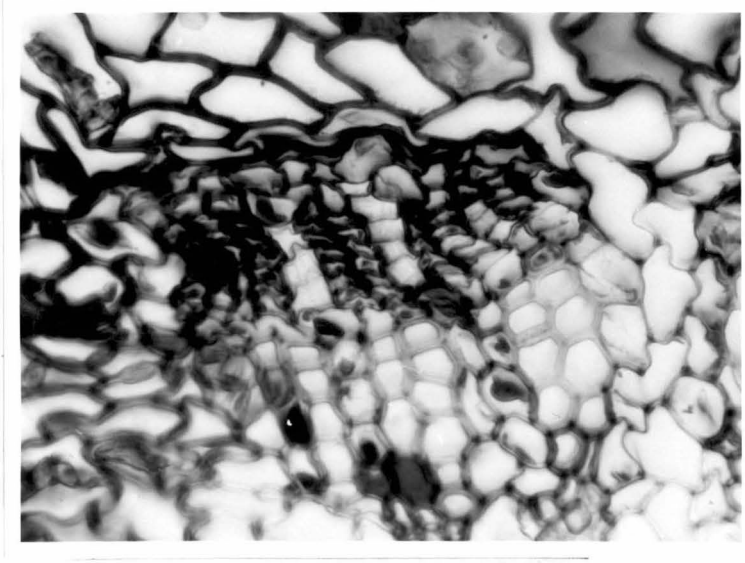
a

(x 98)



b

(x 250)



c

(x 625)

Fig. 14. Transverse section of a day 4 mucus treated needle, from the "black band" region.

a: whole section, with effects of water loss.

b: mesophyll is empty and flaccid, and the stoma is widely opened. Epithelial cells of the resin canal are necrosed.

c: effects of water loss are also apparent in the endodermis and transfusion tissue. The phloem bundles and ray parenchyma cells are only very slightly affected by the mucus treatment. However breakdown of parenchyma cells is quite extensive.

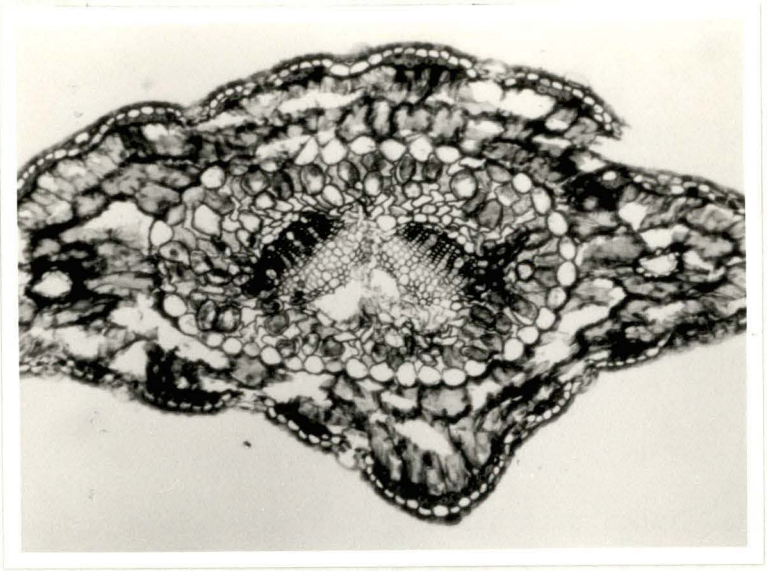
Fig. 15. Longitudinal sections of mucus treated needles, taken from the "black band" regions.

a: 4 days after treatment, showing the presence of both intact and empty, collapsed mesophyll. The phloem also consists of a mixture of both intact collapsed cells.

b: 7 days after treatment. The extent of cellular breakdown and necrosis is more severe than in a day 4 needle.

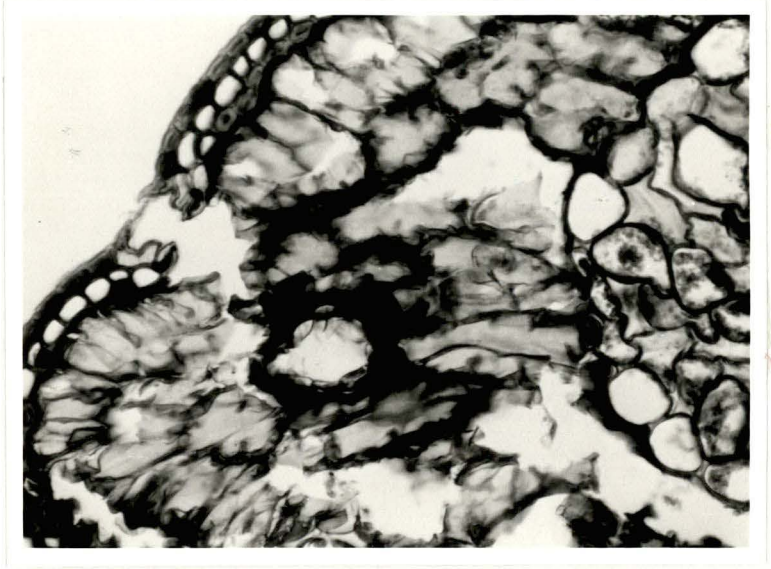
c: 7 days after treatment, showing severe mesophyll breakdown and phloem collapse.

Fig. 14



a

(x 98)



b

(x 250)

c

(x 250)

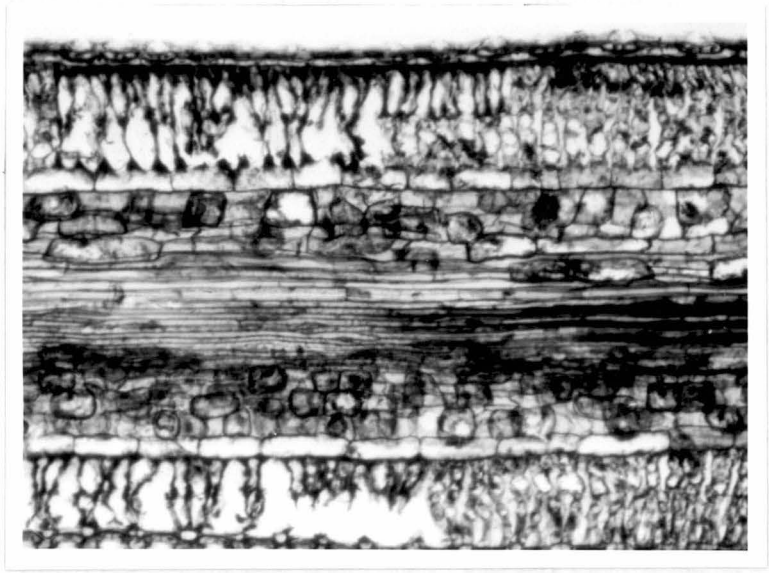
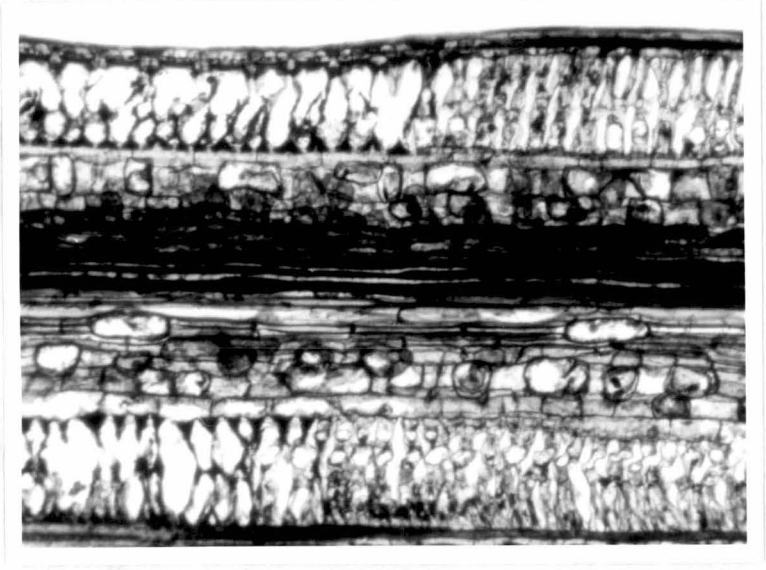


Fig. 15

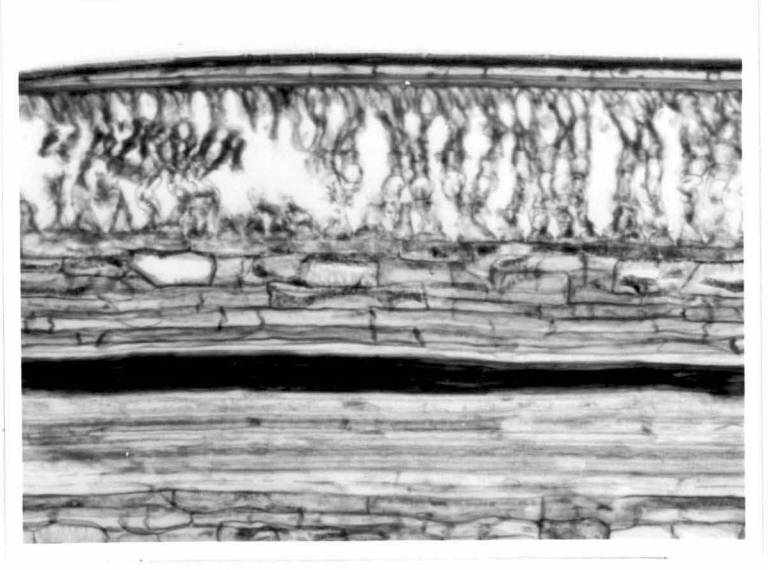
a

(x 98)



b

(x 98)



c

(x 156)

Fig. 16. Longitudinal sections of needles, showing the various forms of tissues.

a: intact and collapsed mesophyll.

b: normal phloem tracheids and transfusion tissue.

c: collapsed phloem tracheids. The refractive, rhomboidal shaped crystals are present in the phloem rays.

Fig. 17. Transverse section of a day 14 mucus treated needle, taken just above the fascicle sheath.

a: whole section appears to be turgid.

b: phloem cells are slightly flattened, and are similar in appearance to the phloem cells of fig. 8. The rest of the tissues show no effects of water loss.

Fig. 18. Transverse section of a day 28 mucus treated needle, taken from within the fascicle sheath.

a: whole section, without effects of water loss. However, a large area of parenchyma cells between the two vascular bundles has broken down.

b: mesophyll cells are intact but sparsely granular. The inner epithelium of the resin canal is hypertrophied and almost occludes the canal.

c: phloem necrosis is almost complete. All of the xylem rays have collapsed, but some of the phloem rays are still intact and are filled with crystals. Spaces between xylem cells are evident (indicated by arrows).

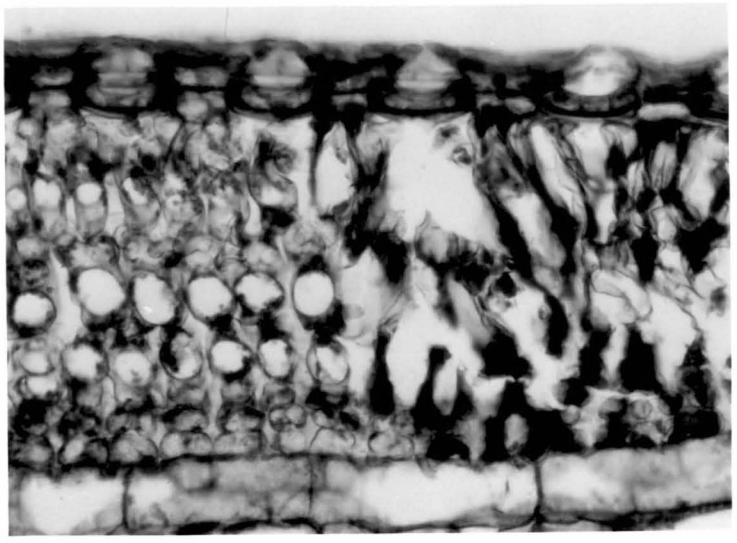
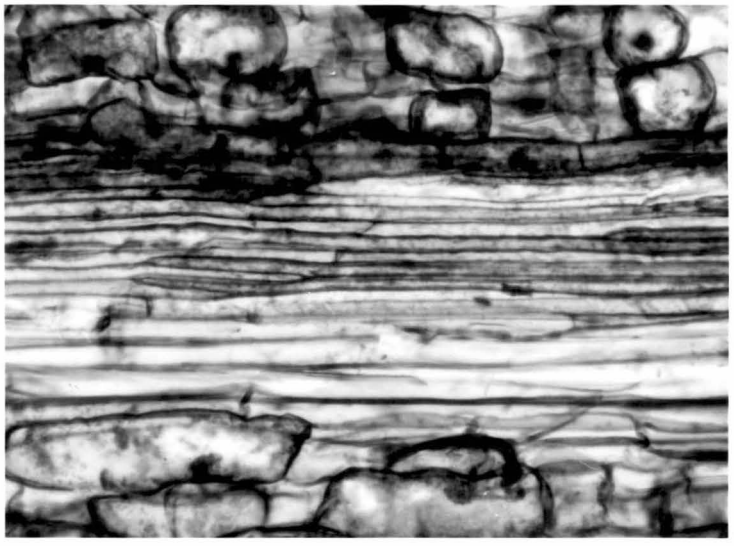


Fig. 16

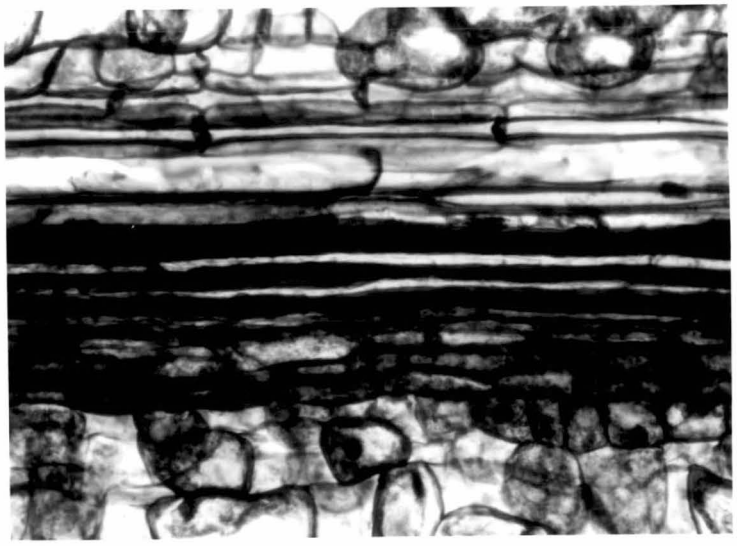
a

(x 250)



b

(x 250)



c

(x 250)

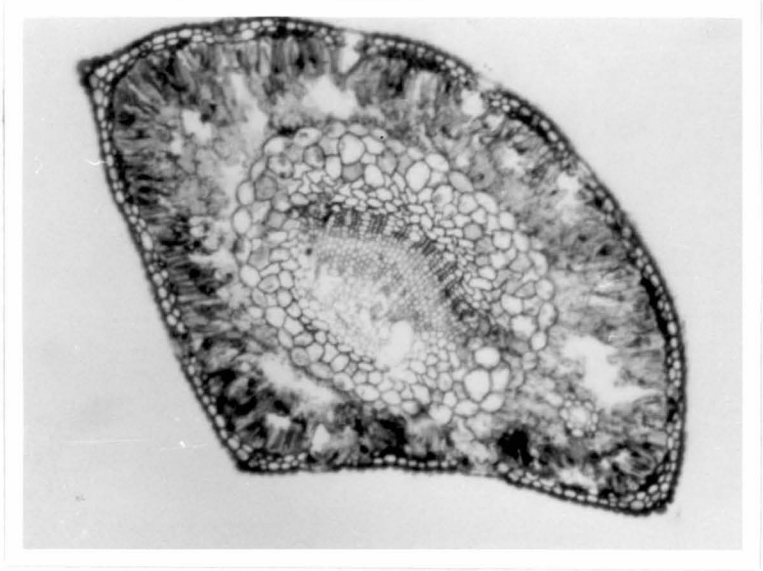
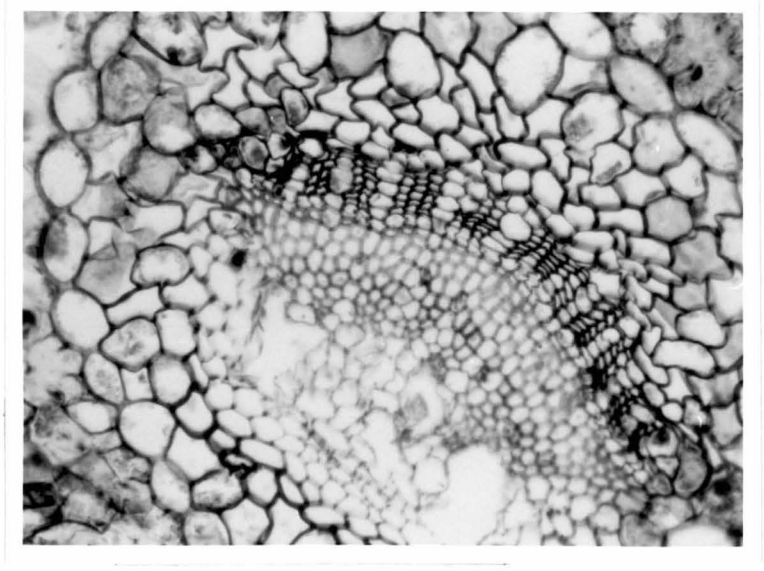


Fig. 17

a

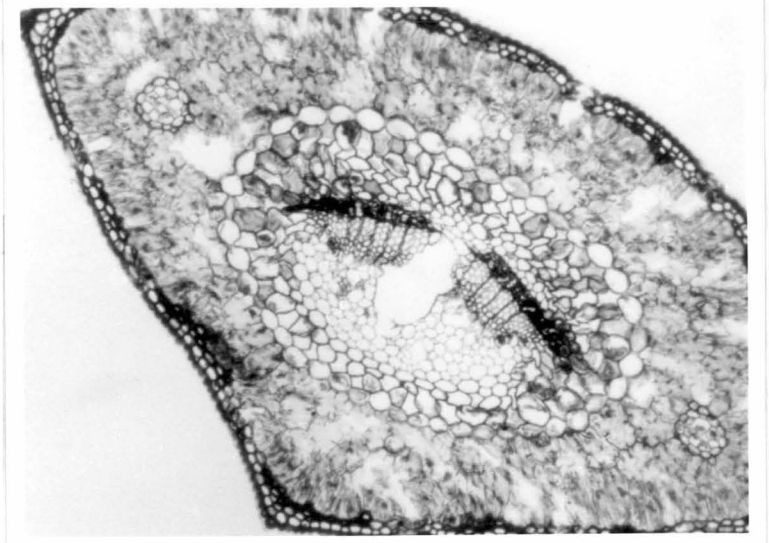
(x 98)



b

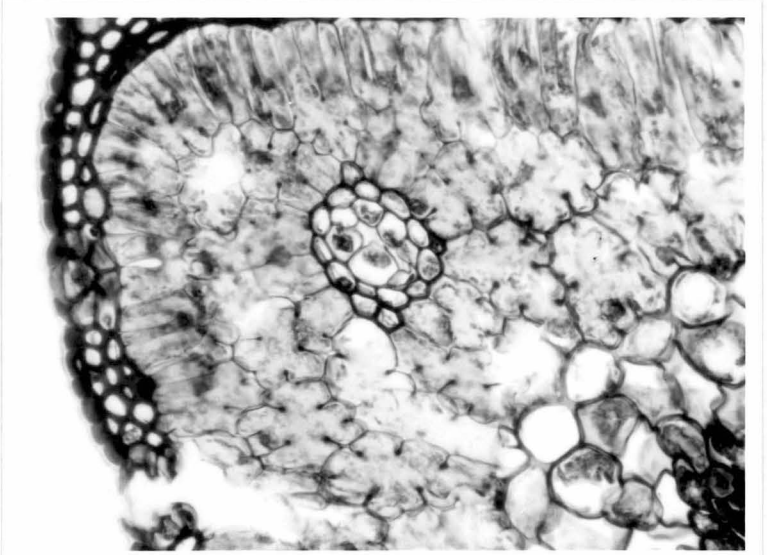
(x 250)

Fig. 18



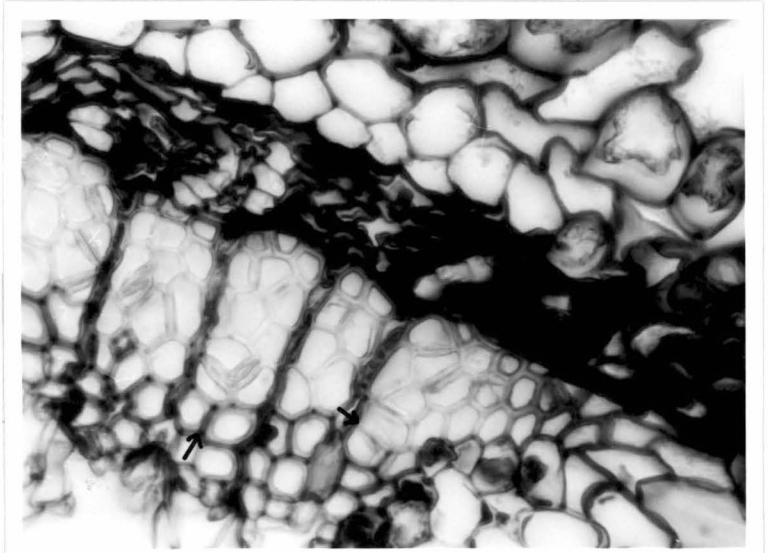
a

(x 98)



b

(x 250)



c

(x 625)

Fig. 19. Transverse section of a day 31 mucus treated needle, taken from the base of the fascicle, just above the region of attachment to the stem.

a: whole section, with a corner of cortical parenchyma being torn away during sectioning. Mesophyll is absent and this part of the needle is non - photosynthetic. A layer of cork cells is present on the inner sides of the triangular shaped needle. The resin canal appears normal.

b: phloem cells have completely necrosed, and some of the xylem rays look squashed.

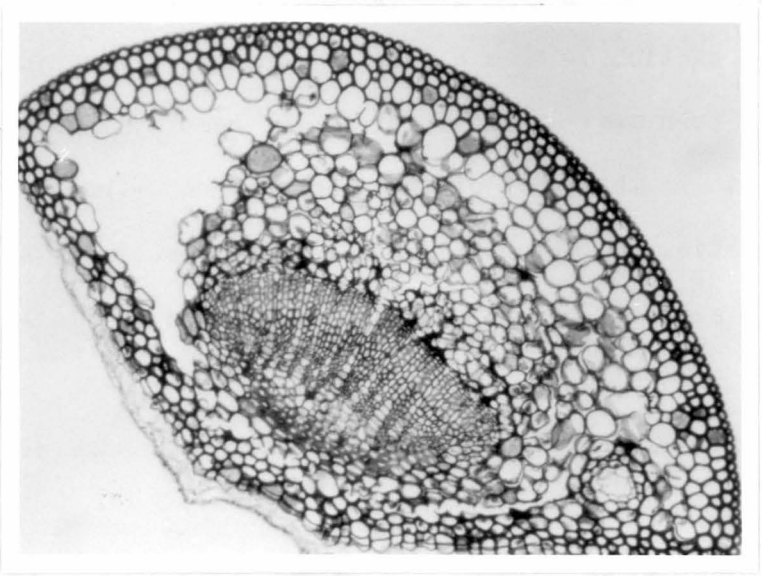
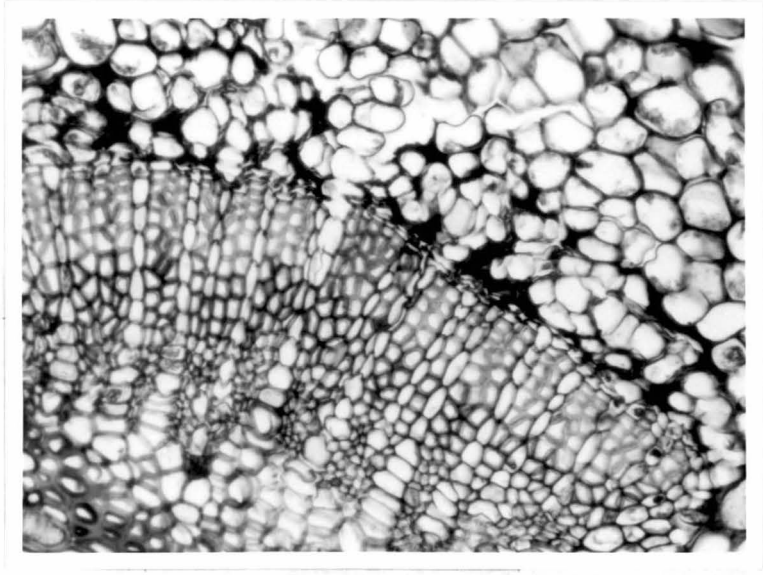


Fig. 19

a

(x 98)



b

(x 250)

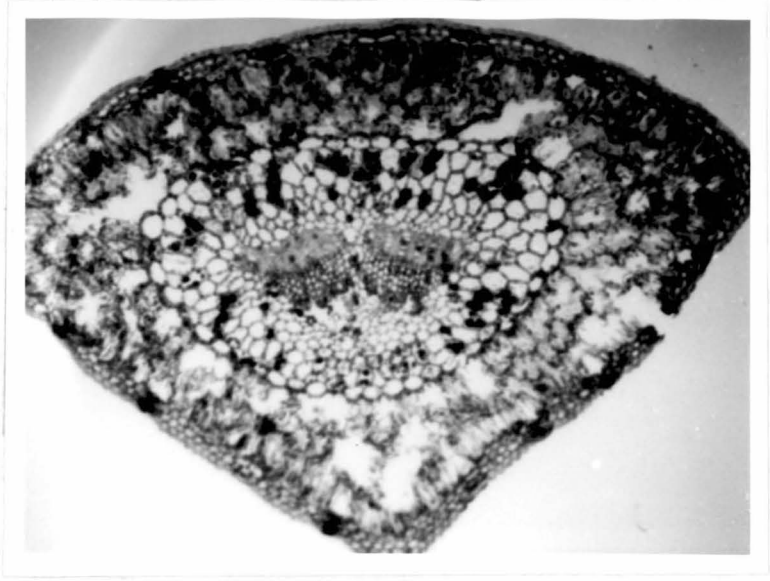


Fig. 20. Transverse section of a day 4, mucus treated needle, showing the distribution of starch. The photomicrograph is at 98 magnifications.

was derived from the base of the needle, the cortex was made up of loosely packed parenchyma cells instead of mesophyll.

The epithelium of the resin canal responded to mucus treatment in two ways: when water loss was excessive and rapid, the epithelium collapsed (figs. 8, and 10 to 14); but when the rate of water loss was slow or minimised, the inner epithelium became hypertrophied and partly occluded the canal (figs. 9 and 18).

Xylem tracheids have rigid lignified walls and did not show any change in cell shape as a result of mucus treatment. However, it was evident in figure 12 that some adjacent xylem cells became separated in the middle lamella as a result of the collapse of the xylem rays. Large areas of pith parenchyma were also broken down (figs. 8 to 10b, 14, 17 and 18).

The distribution of starch in a transverse section of a day 4 mucus treated needle, is illustrated in fig. 20. The starch grains stained purple with I_2/KI and appear as black dots in the photograph. In all of the sections examined so far, it was evident that the transfusion tissue near the phloem contained more starch grains than did the tissue near the xylem.

Physiology and biochemistry

The effects of Sirex mucus on the physiology and biochemistry of P. radiata needles, are shown in graphical form in figures 21 to 28. Anatomical effects were correlated with changes in weight, starch and chlorophyll contents. Losses in fresh weight were recorded for both tree and twig needles, but their dry weights appeared to be also affected by seasonal demands. Whereas the tree and twig needles did not show any gain in dry weight in the autumn, the tree needles in

spring showed an increase.

The change in overall chlorophyll content of autoclaved mucus treated tissues was almost immediate, but a time lag was recorded for tissues treated with fresh mucus. As the tree experiments in spring showed a lag of about four days, and a lag of about nine days in autumn, before showing any significant change to their chlorophyll content, it is suggested that the time lag is dependent on the speed with which the fresh mucus is transported to the needle tissues, and in a form which is readily metabolised by the tissues. Excised twigs in the autumn, which were maintained in an environment of 20 $\pm$ 3°C, showed a time lag of about four days.

As a result of the destruction of chloroplasts, the number of starch grains in the endodermis and the transfusion tissue was also lowered. An anomaly exhibited by excised twigs (both treated and control) is the fall in number of starch grains, in the first two weeks of excision. This anomaly was also apparent in some of the other parameters, and is probably due to physical damage caused by their removal from the tree. The "normal" level of starch was restored after three weeks in the controls, but remained at a low level in the treated twigs.

Mucus treatment caused a significant change to the respiratory activity of needles. The rate of oxygen uptake was about 2½ times higher than normal, in tree needles at day 9; and the respiratory quotient which was maintained at about 1.0 in the controls was about 1.37 in the treated needles, at day 28. Twig needles showed a 60% reduction in their rate of oxygen consumption about five days after excision. Subsequent recovery was gradual in the controls but was

very rapid in the treated needles. Corresponding with the low level of oxygen uptake was the peak in respiratory quotient which reached 1.9 for controls, 3.1 for fresh mucus treated samples and 3.9 for autoclaved mucus treated samples. Recovery after two weeks of excision was indicated by the attainment of the normal value of RQ (i.e., 1.0) in the controls. However, the RQ for treated samples was still high, being 1.5 and 2.1 for fresh and autoclaved mucus treated samples, respectively. The high RQ values are indicative of an anaerobic mode of respiration. Similarly, an enhanced rate of respiration in phloem tissues of the tree trunk, following a Sirex attack, was reported by Madden (1968).

The amount of RNA declined after about three weeks of treatment. The "insoluble" protein content remained virtually unchanged, after a small initial rise, but the levels of soluble protein, peroxidase and amylase were raised and maintained at a high concentration throughout yellowing and up to the time of needle abscission. Soluble protein had been assayed by two different methods, and in both instances, similar trends were observed, thus ruling out the possibility that the increase in soluble protein was due wholly to the increased production of phenols. The soluble proteins also failed to stain with amido black following electrophoresis.

Autoclaved mucus appeared to cause a higher needle peroxidase activity than did fresh mucus in the tree experiments but not in the twig experiments. The peroxidase enzyme consisted of nine anodic and two cathodic bands, of which bands 3, 5 and 6 were particularly conspicuous. Mucus treatment caused an enhancement of all the regular isoenzymes, except those of bands 1 and 2, and the appearance of bands

7 and 8. Photographs of peroxidase electrophoretograms from needle extracts after 0, 7, 16 and 21 days of treatment are given in figure 26, together with interpretative drawings. As the intensity of staining depends on the duration of reaction between enzyme and substrate solution, besides the actual concentration of enzyme present, variations in the staining quality between different electrophoretograms are more likely due to variations in staining time, in this case.

The reduction in starch content was correlated with the rise in amylase activity and soluble sugar content of tree needles. There was no development of new amylase isoenzymes. In twigs, the controls registered a rise in amylase activity with treatment causing an even greater increase, especially about two weeks after excision. The soluble sugar content, after an initial drop in the first week, showed a recovery which then surpassed that of normal values; this change was most noticeable in the controls. Changes in amylase, starch and soluble sugar showed no apparent correlation in the twig needles.

No qualitative differences due to mucus treatment were detected in the "free" sugar complement of needle extracts. The following sugars with their relative amounts indicated, are: glucose (3+), galactose (2+), arabinose (+), mannose (+), and ribose (trace amount).

From investigations carried out so far, it seems that autoclaved mucus is more effective than raw mucus in promoting physiological responses: the onset of chlorophyll destruction was enhanced by about a week, and greater departures from normal were noted for the respiratory mode, peroxidase (only for tree experiments), amylase and soluble sugar content, when compared with fresh mucus treated samples. Moreover, the process of phloem necrosis was also advanced by at least

two days. These results suggest that autoclaved mucus is transported (and therefore metabolised) by the needle tissues at a faster rate than for fresh mucus. Actual measurements of the rates of uptake of autoclaved and fresh mucus solutions in excised twigs have shown this to be so (fig. 29). However, the apparent slowing down in the rate of uptake of autoclaved mucus solution beyond one week of treatment may be due to the senescence processes which would already have begun. The rates of uptake of the mucus solutions were slower than for deionised water.

Similar experiments with trees in which needles of two age groups were sampled, in winter 1968, showed severe dehydration and chlorosis of the two year old needles but only comparatively slight symptomatic responses from the one year old needles. The two year old needles eventually abscissed, but the one year old needles recovered and resumed new growth in the following spring. Figures 27 and 28 are derived from data first used by myself in 1969 (Honours Thesis). Significant differences were observed between needles of the two age groups, these being:

a) maintenance of an aerobic mode of respiration by the treated young needles, but an anaerobic mode was adopted by the old needles.

b) reduced levels of chlorophyll were restored to normal after five weeks of treatment in young needles. During this period, the old needles had turned brown, and their supply had diminished through abscission and usage.

c) the fresh weight was unaltered, but the dry weight was restored to normal after reaching a peak in the first five weeks, in treated young needles. There was an apparent accumulation of

both fresh and dry weights by the old needles.

d) density scans of peroxidase electrophoretograms showed high activities in the old needles at the time of abscission. In young needles, the period of chlorophyll destruction was characterised by the maintenance of high peroxidase activities (at week 5), and subsequent re-greening resulted in lowered enzymic activities (at week 13).

The "old needles" refer to those which are of two years old, and the "young needles" refer to those which are of one year old. Observations so far, seem to indicate a correlation between the mucus - induced chlorosis of needles and a high peroxidase activity, and between age and a high peroxidase activity. Measurements given in table 1 lend further support to this observation.

Table 1: Peroxidase activity of healthy, resistant or susceptible needles, of one or two years old.

Pine needles, after mucus treatment		Peroxidase activity
Age (years)	Description	ΔO.D./min/gm dry tissue
2	healthy, untreated	0.80
1	healthy, untreated	0.52
1	susceptible, yellow	2.05
1	resistant, yellow - green	1.16

As mentioned earlier, age appears to be an important factor in determining resistance or susceptibility of pine needles to mucus treatment. This is undoubtedly due to the physiological status of

the tissues, and in turn, is related to the level of endogeneous hormones present (refer to literature on plant senescence). Use of kinetin in delaying the development of senescence symptoms in radish leaf tissues, and the stimulating effect of Sirex mucus on its senescence, was reported in my Honours Thesis (Fong, 1969), and shown in table 2.

Table 2: Effect of kinetin and Sirex mucus on the development of chlorosis in radish leaf discs (1cm diameter), in darkness.

Test solutions	Time taken for development of chlorosis (days)
Deionised water	10
Kinetin, 10mg/l	15
Mucus, 0.5mg/ml	3
Mucus (0.5mg/ml) + kinetin (10mg/l) (1 : 1, v/v)	14

In addition to the enhanced yellowing of radish leaf discs, there was the development of large opaque areas which indicated tissue breakdown. Thus, kinetin effectively delayed the onset of senescence of radish leaf tissues, although it was not possible for the cytokinin to prevent senescence, in the abscence of nutrient supplement and sunlight. This experiment also indicates the non - specificity of Sirex mucus in causing tissue senescence, although Coutts (1969b) suggested the contrary when he found that the mucus had no effect on Populus seedlings and Eucalyptus saplings.

Using 5mm lengths of pine needles, it was also possible to observe

Fig. 21. Effect of Sirex mucus treatment on levels of fresh weight, dry weight, oxygen uptake and respiratory quotient, in P. radiata needle segments of trees (in spring) and twigs (in autumn). ——— Control

-o- -o- Treated with raw mucus

·-▲-·-▲- Treated with autoclaved mucus

The P values are as follows:

		Period (days) where comparisons are made	Control needles	Needles treated with mucus	
				Raw	Autoclaved
Trees	Fresh weight	-14 to 0 and 14 to 28	0.01	< 0.001	-
Twigs	Fresh weight	0 to 7 and 21 to 28	0.8	< 0.001	0.001
Trees	Dry weight	-14 to 0 and 4 to 14	0.02	< 0.001	-
Twigs	Dry weight	0 to 7 and 16 to 28	0.5	0.8	0.3
Trees	Oxygen uptake	-21 to 0 and 7 to 17	0.01	< 0.001	-
Twigs	Oxygen uptake	9 to 11 and 16 to 21	0.05	0.01	0.02
Trees	Respiratory quotient	-21 to 0 and 7 to 17	0.8	0.001	-
Twigs	Respiratory quotient	0 to 2 and 4	0.2	0.01	0.01

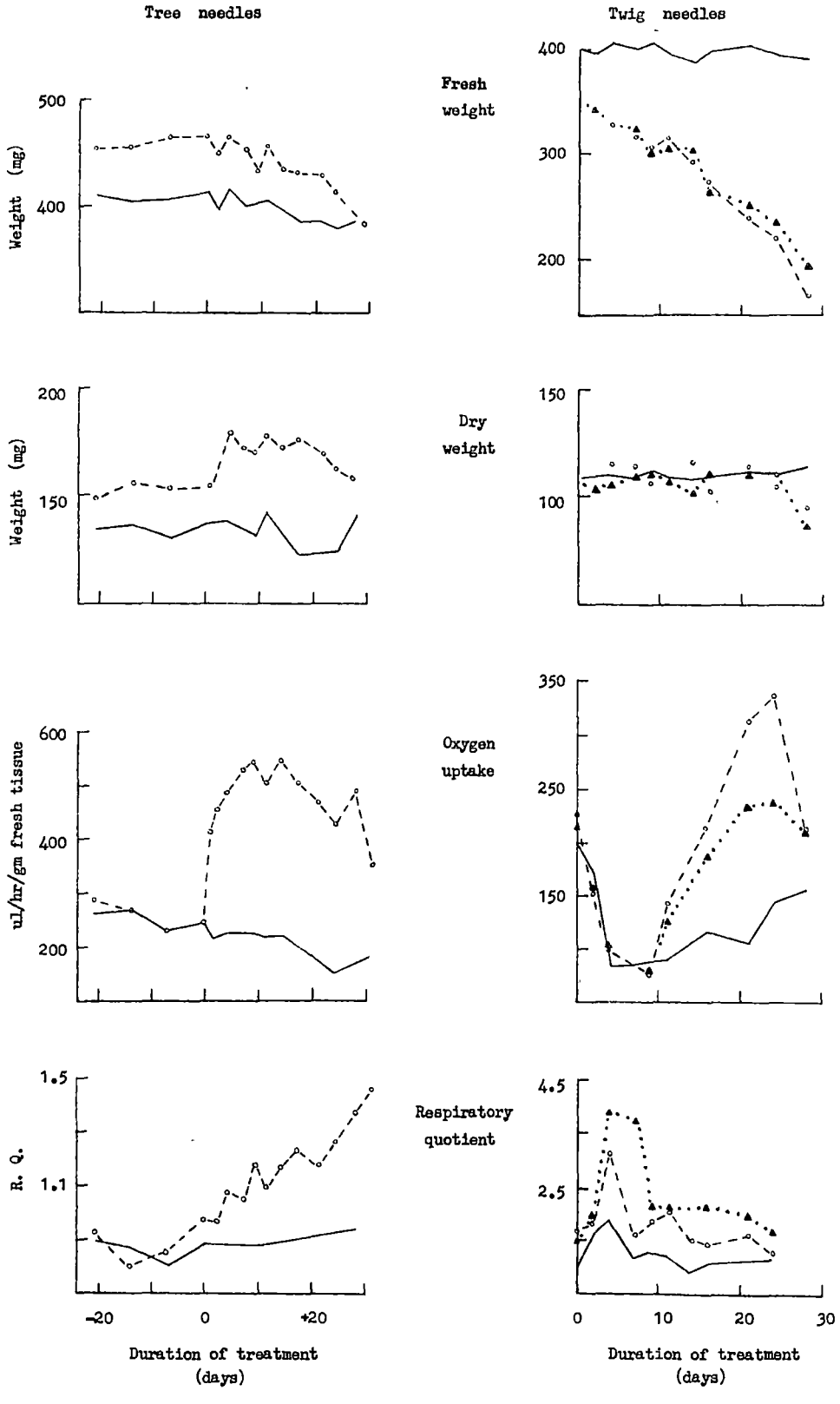

Tree needles
Twig needles
Fresh weight
Dry weight
Oxygen uptake
Respiratory quotient
Weight (mg)
ul/hr/gm fresh tissue
R. Q.
500
400
200
150
600
1.5
1.1
400
300
100
350
250
4.5
2.5
-20
0
+20
10
20
30
Duration of treatment (days)
Duration of treatment (days)

Fig. 21

Fig. 22. Effect of *Sirex* mucus treatment on levels of chlorophyll, starch, amylase and soluble sugars, in *P. radiata* needle segments of trees (in spring) and twigs (in autumn).

——— Control — o — o — Treated with raw mucus

. . . . Treated with autoclaved mucus

The P values are as follows:

		Period (days) where comparisons are made		Control needles	Needles treated with mucus	
					Raw	Autoclaved
Trees	Chlorophyll$_a$ (i.e., C_a)	-21 to 0				
		and	14 to 28	0.3	< 0.001	-
Trees	Chlorophyll$_b$ (i.e., C_b)	-21 to 0				
		and	14 to 28	0.8	< 0.001	-
Twigs	Total chlorophyll	0 to 4				
		and	21 to 28	0.5	< 0.001	< 0.001
Trees	Starch	-7 to 0				
		and	21	0.2	0.05	-
Twigs	Starch	2 to 9				
		and	19 to 28	0.01	-	0.7
Trees	Amylase	-14 to 0			0.01 to	
		and	1 to 4	0.2	0.001	-
Trees	Amylase	-14 to 0				
		and	14 to 28	0.02	0.001	-
Twigs	Amylase	0 to 7				
		and	14 to 16	0.001	0.001	< 0.001
Trees	Soluble sugars	0 to 4		0.9	0.1	-
		and	11 to 28	0.9	0.1	-
Twigs	Soluble sugars	4 to 7				
		and	14 to 16	0.02	< 0.001	0.001

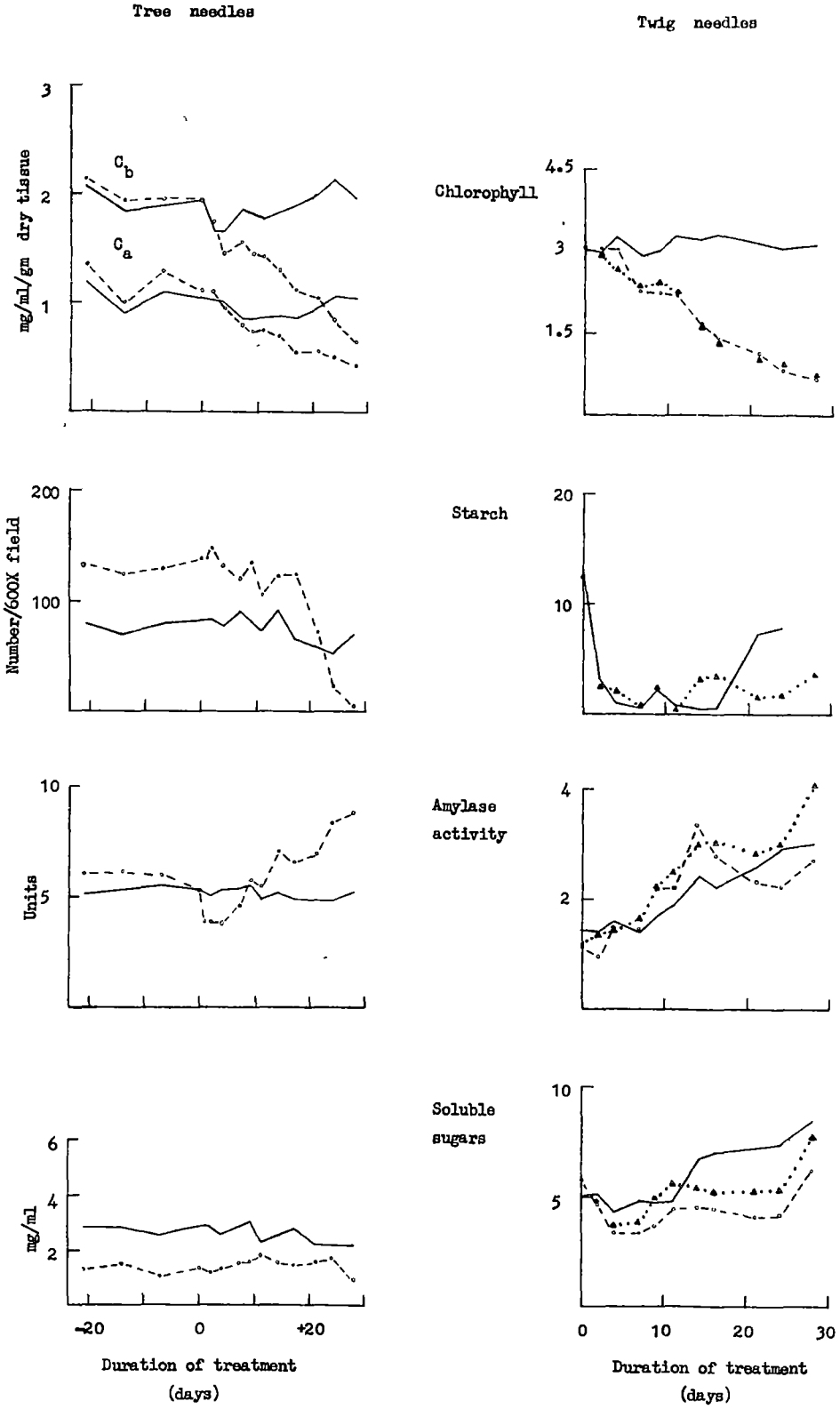

Tree needles
Twig needles
mg/ml/gm dry tissue
3
2
1
C_b
C_a
Chlorophyll
4.5
3
1.5
Number/600X field
200
100
Starch
20
10
Units
10
5
Amylase activity
4
2
mg/ml
6
4
2
Soluble sugars
10
5
-20
0
+20
0
10
20
30
Duration of treatment (days)
Duration of treatment (days)

Fig. 22

Fig. 23. Effect of Sirex mucus treatment on levels of soluble protein (as determined by the two different procedures, with the Biuret or Folin-Ciocalteau reagents) and peroxidase, in P.radiata needle segments of trees (in spring) and twigs (in autumn). ——— Control

-o- -o- Treated with raw mucus

·A· ·A· Treated with autoclaved mucus

The P values are as follows:

		Period (days) where comparisons are made	Control needles	Needles treated with mucus	
				Raw	Autoclaved
Trees	Soluble protein (with Folin-Ciocalteau reagent)	-14 to 2 and 11 to 25	0.02	<0.001	-
Twigs	Soluble protein (with Folin-Ciocalteau reagent)	0 to 2 and 11 to 16	0.02	0.05	0.001
Trees	Peroxidase	-21 to 0 and 7 to 21	0.02	<0.001	-
Twigs	Peroxidase	0 to 2 and 21 to 28	0.01	0.001	0.001

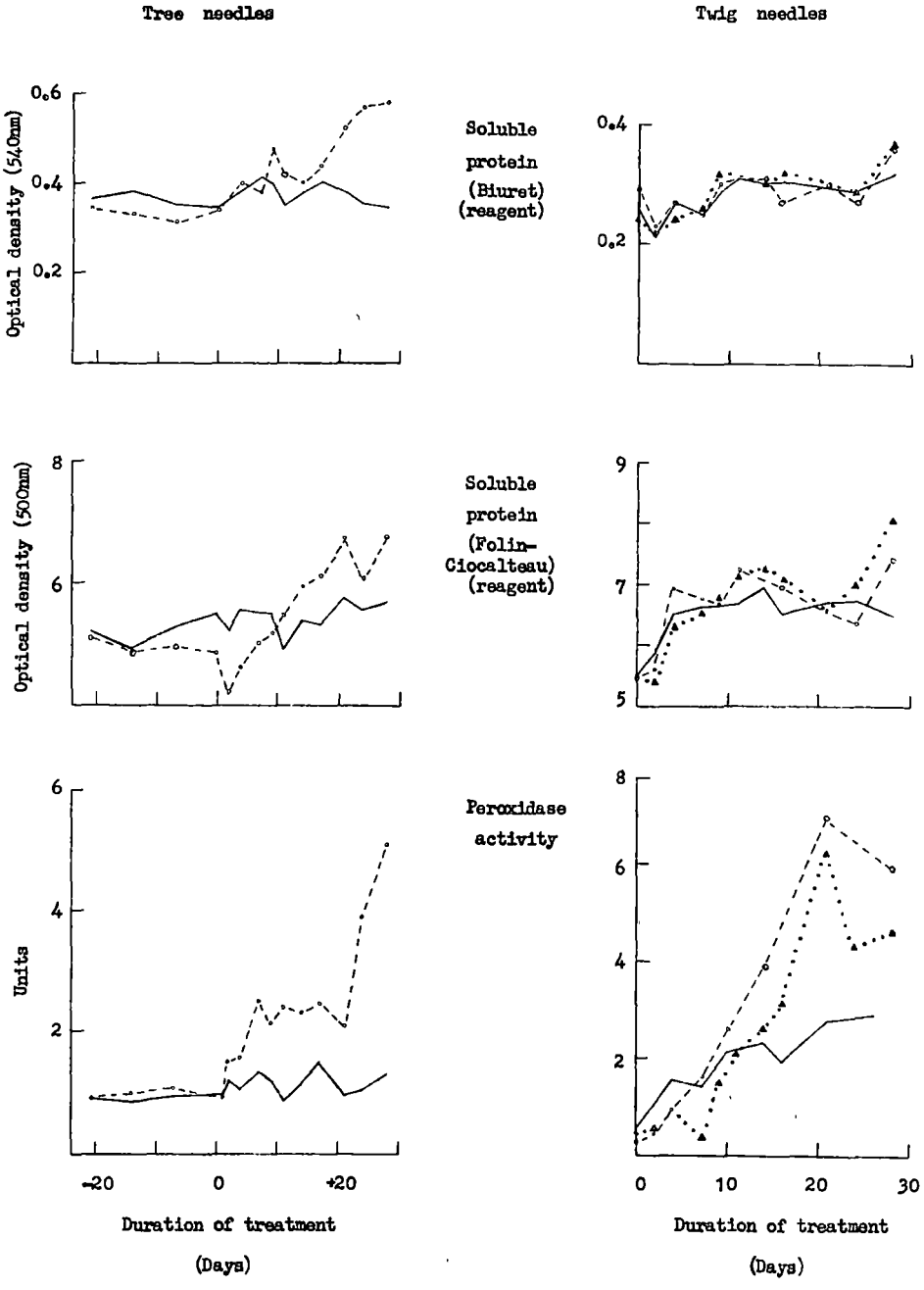

Tree needles
Twig needles
Optical density (540nm)
0.6
0.4
0.2
Soluble protein (Biuret) (reagent)
0.4
0.2
Optical density (500nm)
8
6
Soluble protein (Folin-Ciocalteau) (reagent)
9
7
5
Units
6
4
2
Peroxidase activity
8
6
4
2
-20
0
+20
0
10
20
30
Duration of treatment
(Days)
Duration of treatment
(Days)

Fig. 23

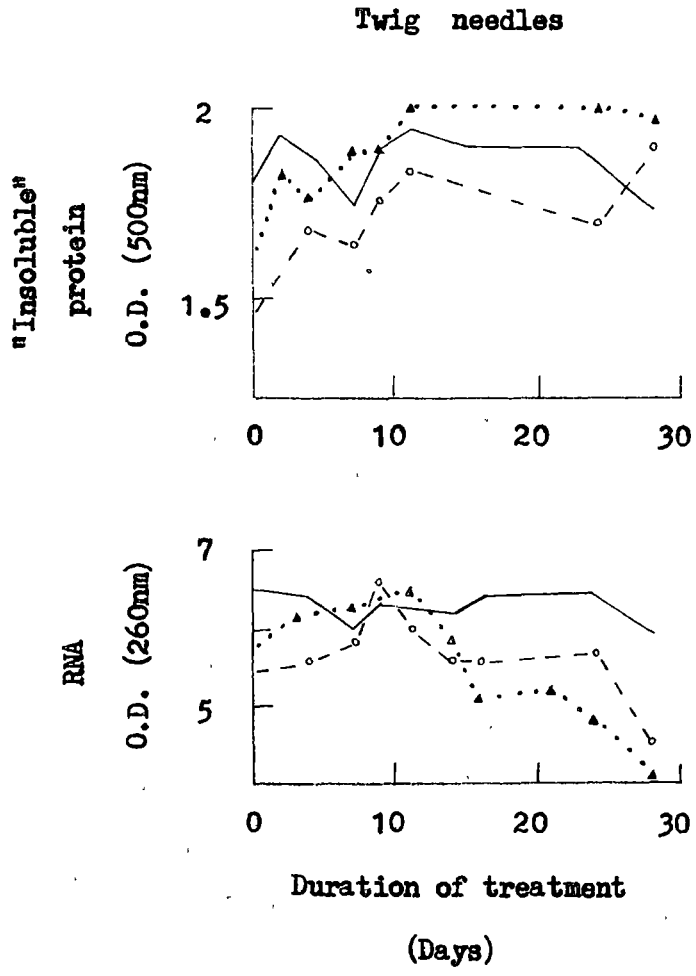


Fig. 24. Effect of Sirex mucus treatment on levels of "insoluble" protein and RNA, in P.radiata needle segments of trees (in spring) and twigs (in autumn).

——— Control

–o– –o Treated with raw mucus

.▲. .▲. treated with autoclaved mucus

Fig. 25. Effect of Sirex mucus treatment on levels of fresh weight, dry weight, chlorophylls a and b, and peroxidase, in P.radiata needle segments, of trees in autumn.

——— Control -o- -o- Treated with raw mucus

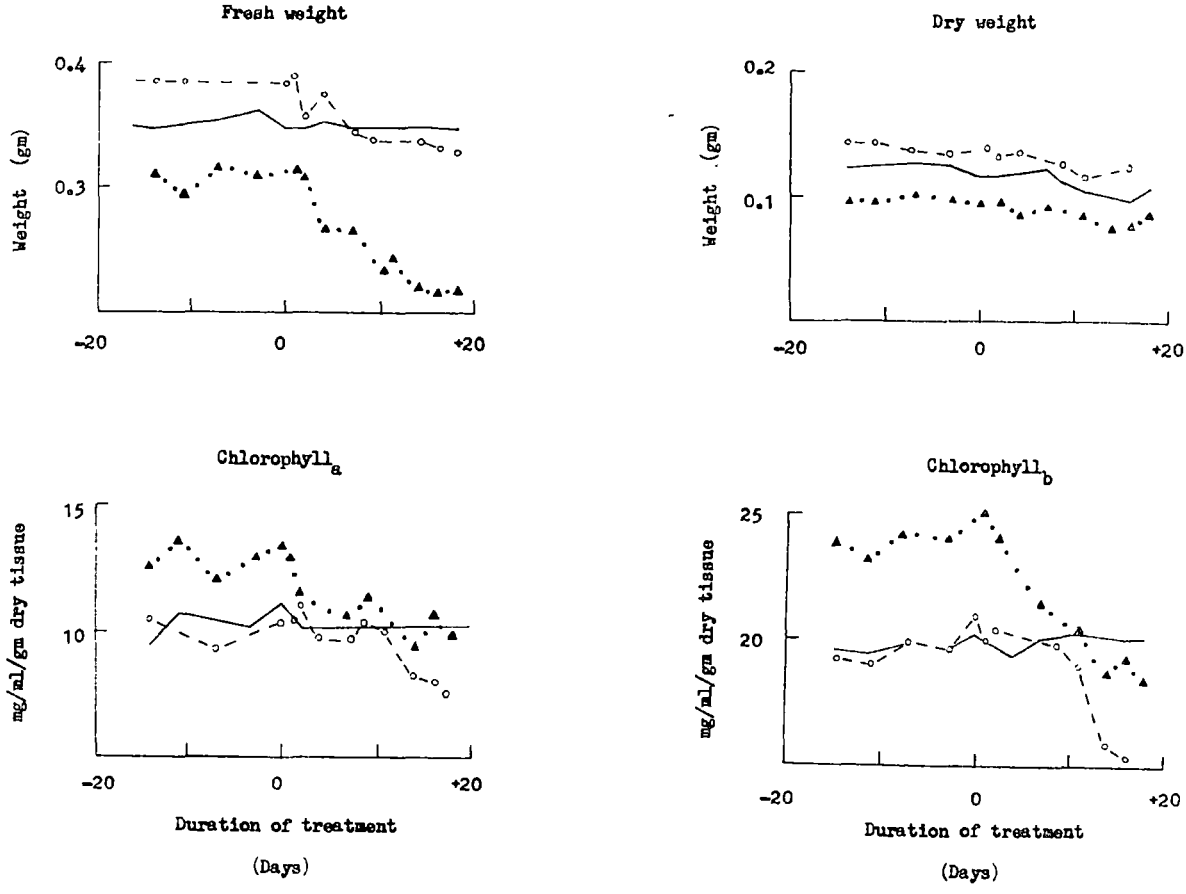
·▲· ·▲· Treated with autoclaved mucus

Fig. 26. Photographs and some interpretative drawings of peroxidase electrophoretograms, from extracts of P.radiata needle segments, after treatment with raw mucus (M) or with de-ionised water (C). The peroxidase isoenzymic pattern is similar for needle extracts from twigs or trees that have been treated with raw or autoclaved mucus.

Fig. 27. Effect of Sirex mucus treatment on levels of fresh weight, dry weight, oxygen uptake, respiratory quotient and chlorophyll, in P.radiata needle segments, of trees in winter. Two year old (old) and one year old (young) needles were used. This data is taken from my Honours Thesis (Fong, 1969).

——— Control -o- -o- Treated with raw mucus

Fig. 28. Density scans of peroxidase electrophoretograms, from extracts of P.radiata needle segments, of trees in winter. Two year old (old) and one year old (young) needles were used. This data is taken from my Honours Thesis (Fong, 1969).


Fresh weight
Weight (gm)
0.4
0.3
-20
0
+20
Dry weight
Weight (gm)
0.2
0.1
-20
0
+20
Chlorophyll a
mg/ml/gm dry tissue
15
10
-20
0
+20
Duration of treatment
(Days)
Chlorophyll b
mg/ml/gm dry tissue
25
20
-20
0
+20
Duration of treatment
(Days)

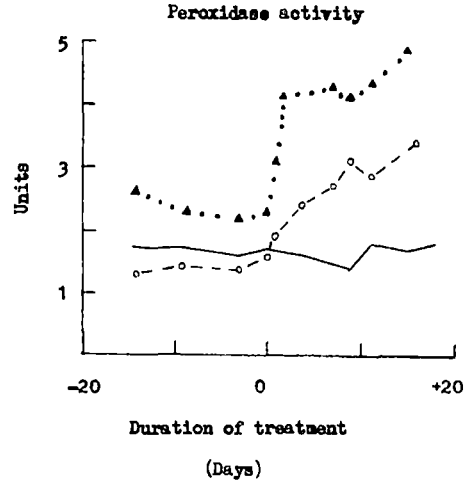
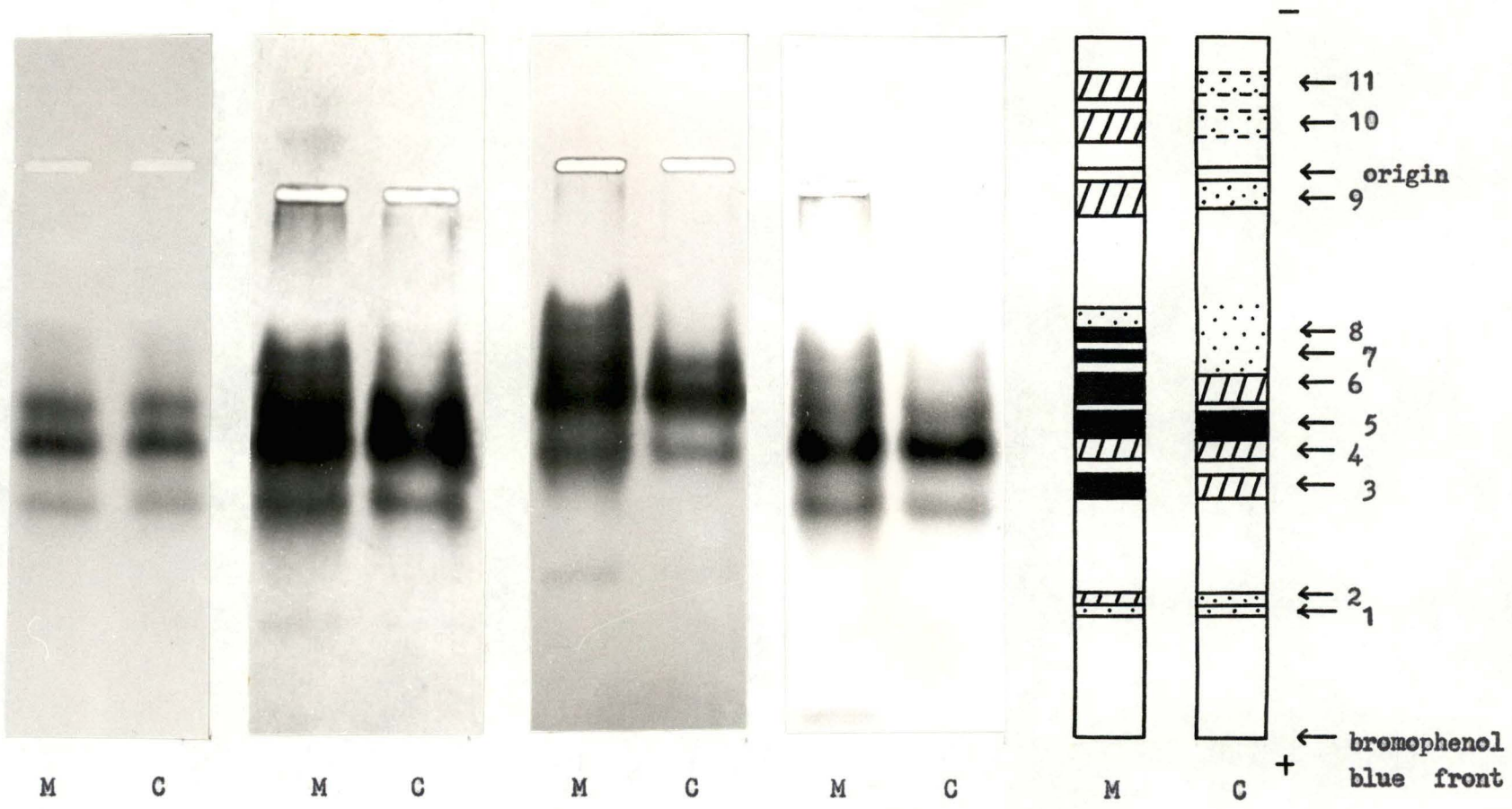

Peroxidase activity
Units
5
3
1
-20
0
+20
Duration of treatment
(Days)

Fig. 25



Duration of treatment: 0 days | 7 days | 16 days | 21 days | 7 days

Figure 26

Notations:

■ ▨ ⁘ for decreasing intensity of stain, from left to right.

\+ Anode, − Cathode.

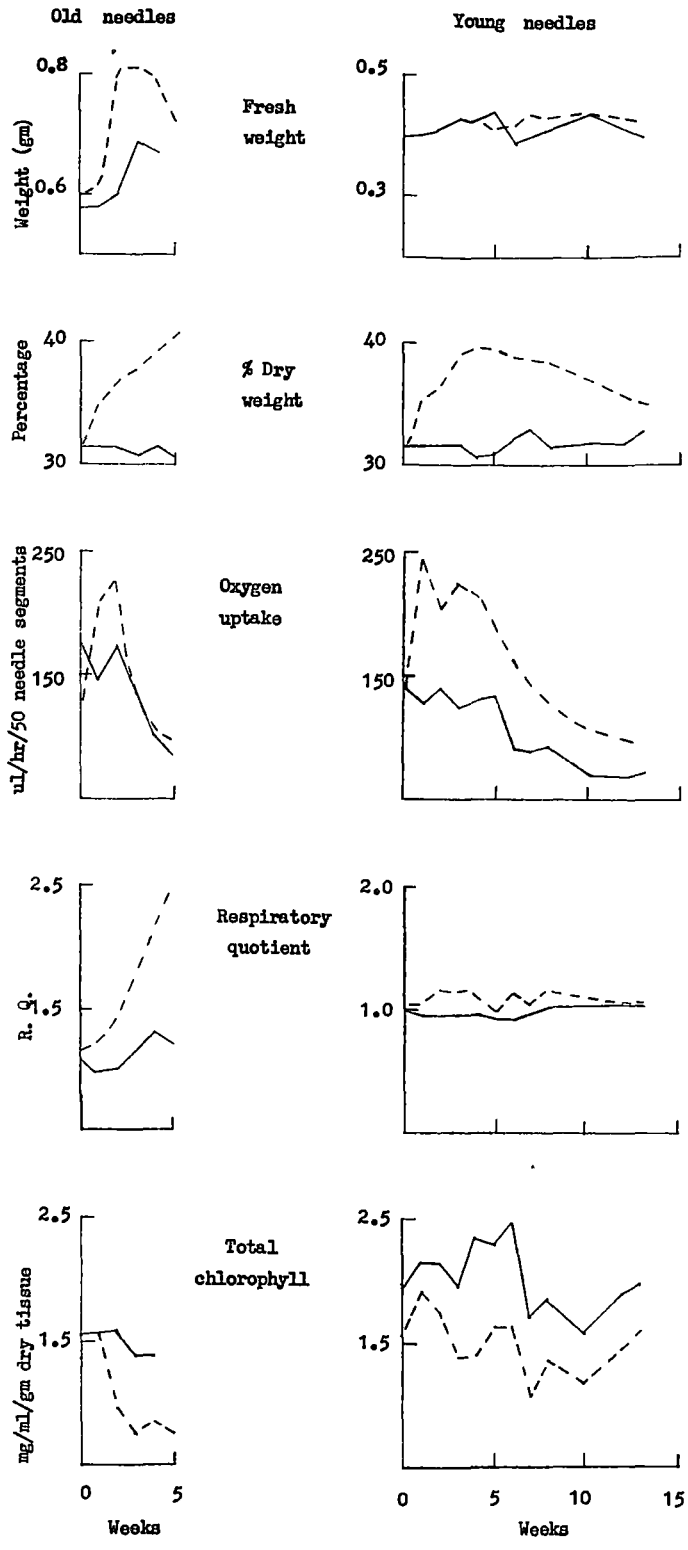

Old needles
Young needles
Fresh weight
Weight (gm)
0.8
0.6
0.5
0.3
% Dry weight
Percentage
40
30
Oxygen uptake
ul/hr/50 needle segments
250
150
Respiratory quotient
R. Q.
2.5
1.5
2.0
1.0
Total chlorophyll
mg/ml/gm dry tissue
0
5
10
15
Weeks

Fig. 27

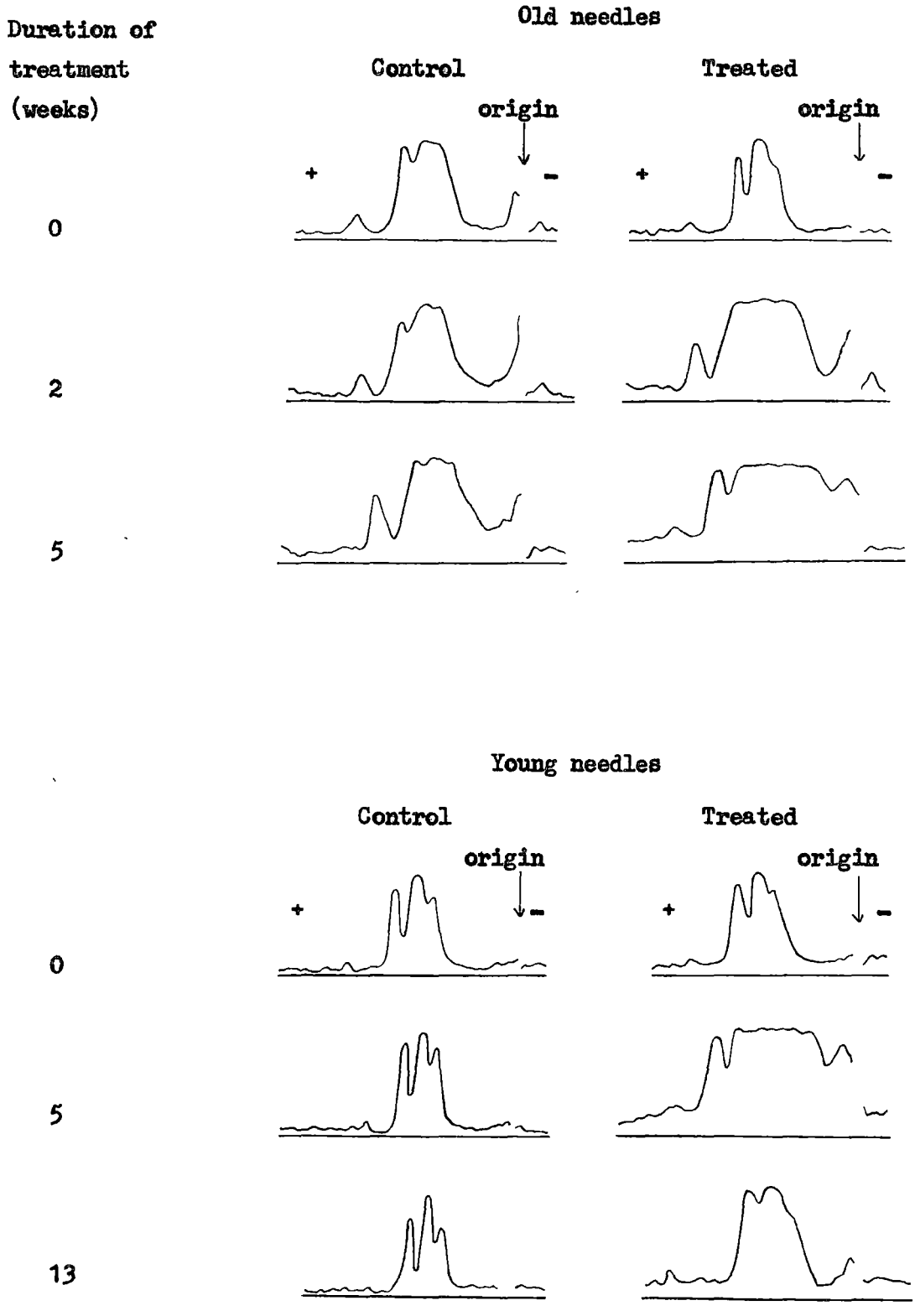

Old needles
Duration of treatment (weeks)
Control
Treated
origin
origin
+
-
+
-
0
2
5
Young needles
Control
Treated
origin
origin
+
-
+
-
0
5
13

Figure 28

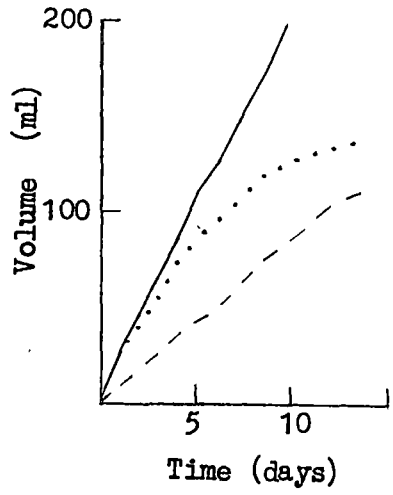


Fig. 29. Uptake of solutions by excised twigs of Pinus radiata, under constant phytotron conditions.

——— deionised water

· · · · autoclaved mucus solution (0.05%)

- - - - raw mucus solution (0.05%)

the senescence - retarding effect of kinetin in the presence of de-ionised water or Sirex mucus. However, as the thick epicuticular wax on the needles had minimised surface absorption of mucus, the senescence effects were observed only on the cut ends of the needles.

Discussion

P.radiata needles responded to mucus treatment with a range of symptoms which were quite similar in certain respects, to both natural and disease - induced senescence of other plant foliage. The mucus - induced senescence of P.radiata resulted in reduced levels of chlorophyll, fresh weight, starch, RNA, but increased levels of amylase, peroxidase and soluble protein, a temporary rise in the respiratory rate which then declined, and an alteration to the mode of respiration. Natural leaf senescence of other plants is generally accompanied by losses of chlorophyll, protein and RNA, together with reduced rates of photosynthesis and respiration in Perilla frutescens (Hardwick and Woolhouse, 1967; Hardwick, et al, 1968); loss of starch from tobacco (Matheson and Wheatley, 1962); loss of fresh weight but rise in the peroxidase activity of cucumber cotyledons (Lewington, et al, 1967); and unaltered levels of DNA in Phaseolus vulgaris (Phillips and Fletcher, 1969). In P.radiata, these physiological and biochemical changes are accompanied by dehydration and necrosis of the tissues, which result in starvation.

The primary histological effects of Sirex mucus include the destruction of chloroplasts, necrosis of phloem tracheids and ray parenchyma, and maintenance of a high proportion of opened stomata. As a result of excessive water loss through the opened stomata, unlignified tissues (and the slightly lignified transfusion tissue)

became flaccid and eventually collapsed. Protection from dehydration was derived from the fascicle sheath, as tissues from the ensheathed region showed no other signs of cellular distortion even when phloem and ray parenchyma necrosis and chloroplast destruction had occured. In the absence of rapid and excessive water loss, the needles underwent a more gradual rate of senescence and appeared to contain resin canals which were smaller than normal, due to the swelling of the inner epithelium. Enlargement or hypertrophy of epithelial cells of the resin canals is one symptom of natural or environmentally - induced senescence of four other Pinus species and of Pseudotsuga menziesii (Stewart, et al, 1973); other symptoms being the collapse of mesophyll and endodermis. Unlike the mucus effect, however, exposure of these conifers to drought or toxic chemicals caused the transfusion and phloem tissues to become hypertrophied and granulated before finally collapsing. Phloem necrosis is a common disease of some commercially important plants. Mature plantation trees of Coffea liberica which became infected with Phytomonas leptovasorum flagellates in their phloem vessels underwent phloem hyperplasia and then breakdown (Vermuelen, 1968). The stems of blueberry plants which were susceptible to Botryosphaeria corticis had completely disorganised cortex and phloem and hypertrophied xylem rays (Milholland, 1970). As a result of the yellow - dwarf virus infection, barley plants were stunted and chlorotic, but the leaves were found to contain high levels of starch, soluble carbohydrates and reducing sugars and low levels of soluble nitrogen (Orlob and Arny, 1961), probably due to the degeneration of phloem (Esau, 1957).

Yellow, mucus susceptible pine needles had a low starch content

but high amylase activity; an observation similarly reported for TMV - infected young tobacco leaf discs (Doke and Hirai, 1969). Concomitant to the stimulation of starch degradation by high amylase activities, the TMV - infected tobacco leaf discs also incorporated less $^{14}CO_2$, which suggested reduced starch synthesis. Cabbage cotyledons detached from the main plant showed a gradual decline in chlorophyll content, the rate of which was not affected by infection with Peronospora parasitica, but the respiratory rate became twice that of the uninfected control, just prior to sporulation (Thornton and Cooke, 1974). In barley leaves infected with powdery mildew, the chlorophyll content and photosynthetic activity of resistant leaves were not affected and their respiration showed only a slight increase; however, susceptible leaves experienced a slight increase in photosynthesis 48hr after inoculation which subsequently declined, the chlorophyll content was then reduced but respiration increased (Scott and Smillie, 1966).

The respiratory peak of P.radiata needles was reached in the first week of mucus treatment, before severe tissue breakdown and chlorosis had occured. In susceptible needles there was an accompanying increase in the expiration of CO_2, which resulted in values of RQ being greater than one. Needles which did not adopt this anaerobic mode of respiration inspite of a high respiratory rate, subsequently recovered from mucus treatment. Injury caused by their removal from the tree also resulted in a temporary fall in the rate of respiration and a small corresponding rise in RQ, in twig needles. High respiratory rates in the early phases of infection, prior to the onset of degradative processes in the host plant, appears to be a com-

mon effect (Wood, 1967), and observed in such host - parasite interactions as mildewed barley and rusted wheat leaves (Shaw and Samborski, 1957); Fusarium infected tomato leaves (Collins and Scheffer, 1958); barley affected by the yellow - dwarf virus (Orlob and Arny, 1961); and Pseudomonas affected tobacco leaves (Németh and Klement, 1967). In contrast to the mucus effect, the fungus affected barley and wheat leaves adopted an aerobic mode of respiration when RQ values fell from 1 to 0.8 - 0.85, after the attainment of the respiratory peak (Shaw and Samborski, 1957). This type of intensive oxidative metabolism by the host plant may be an important pre-requisite for the development of the parasite, often leading to the production of compounds which then become toxic to the host (therefore a susceptible or hypersensitive reaction) or to the parasite (therefore a resistant reaction) (Kiraly and Farkas, 1959).

There is little evidence to prove that peroxidase participates in the respiratory activity of diseased tissues. The rise in enzymic activity may be associated with available precursors (i.e., protoporphyrin) for peroxidase synthesis, due to the declining production of chlorophyll (Burris, 1960). Age and mucus - induced chlorosis of P.radiata needles were correlated with high peroxidase activities; the overall quantitative and qualitative changes are probably due mainly to senescence effects, although Titze (1965) found that susceptible buds of P.radiata contained lower peroxidase and phenoloxidase activities than did the resistant buds. Farkas and Kiraly (1958) suggested that the rise in peroxidase activity may be a self - regulating detoxification process whereby H_2O_2 accumulation is being eliminated. Tissues which showed natural or artificially - induced resis-

tance to infection also contained high peroxidase activities (Stahmann, et al, 1966; Fehrmann and Dimond, 1967; Weber, et al, 1967; Lovrekovich, et al, 1968; Macko, et al, 1968; Curtis, 1971), and peroxidase was shown to inhibit the in vitro development of fungal mycelia (Macko, et al, 1968). A high polyphenoloxidase activity was also associated with resistance to Ceratocystis fimbriata, in sweet potato tissues (Weber, et al, 1967).

One response of P.radiata sapwood to mechanical injury or to Sirex attacks, was the production of ethylene (Shain and Hillis, 1972). Trees which were capable of producing more ethylene also produced more polyphenols, and the production of ethylene occured before the production of polyphenols. Polyphenols were shown to be toxic to the fungus Amylostereum (Coutts and Dolezal, 1966). Exposure of naturally susceptible sweet potato tissues to low concentrations of ethylene also induced resistance to black rot and caused a stimulation to the peroxidase activity (Stahmann, et al, 1966); the stimulated peroxidase activity was shown to have arisen out of active synthesis (Shannon, et al, 1971). However, not all tissues responded to ethylene treatment in the same manner. Whilst the activities of peroxidase and polyphenoloxidase in sweet potato were stimulated by ethylene, only polyphenoloxidase was stimulated in potato tubers and parsnip roots, and none of these enzymes was affected by ethylene in carrot, radish or turnip (Stahmann, et al, 1966). A high peroxidase activity associated with the resistance of tomato leaves to Septoria lycopersici, was also obtained with exposure of the plants to low light intensities or to light of specific wavelengths (Benedict, 1972).

Contrary to the reported association between a high peroxidase

activity and resistance to diseases, Jennings, et al (1969) suggested that the increased activities of peroxidase and phenoloxidase were associated with susceptibility of maize leaves to Helminthosporium carbonum. Wood (1971) also showed that infected, susceptible leaves which developed pronounced symptoms with the cucumber mosaic virus, contained higher peroxidase activities than did the resistant variety which was practically symptomless; and the isoenzymic pattern between the detached senescing leaves and virus infected leaves were also different. Grzelinska (1970) using Fusarium infected tomato leaves, found no quantitative correlation in the peroxidase activity between naturally senescing and diseased plants. He suggested that the change in peroxidase activity following infection was the result of a disturbed metabolism rather than a defense reaction. The high peroxidase and polyphenoloxidase activities produced by Fusarium infected tomato plants appeared to parallel the increased production of ethylene, which reached a maximum when the plants were severely wilted and were about to absciss (Gentile and Matta, 1975). Clearly, ethylene does not appear to be a resistance factor in Fusarium infected tomato plants as it enhances disease development.

The level of RNA in pine needles was noticeably reduced after more than half of the chlorophyll had been destroyed. Thus, the rise in amylase, peroxidase and cold - water - soluble protein may be the result of degradative processes occuring in the senescing needles, rather than the result of active synthesis. The "insoluble" protein level showed a small insignificant rise in the first week of treatment, and is probably related to the reduced rate of translocation from the needles, due to phloem necrosis. However, an altered mode

of metabolism probably accounted for the stimulation in aromatic aminoacid synthesis, in tobacco plants infected with Pseudomonas solanacearum (Pegg and Sequeira, 1968).

Having discussed some of the changes which occur in P.radiata needles which are susceptible to Sirex mucus, and compared them with the changes which occur in other pathogen - infected plants, it is felt that certain physiological and biochemical differences between the pine needle response to Sirex mucus and the plant response to pathogenic infection, may be related to the different basic requirements imposed on the host plant by the invading organisms. The Sirex larvae and Amylostereum fungus have been shown to develop most successfully from a weakened and starving host tree (Coutts and Dolezal, 1966a; Madden, et al, 1964/65, C.S.I.R.O. Report), with the mucus acting as a "tree - conditioning agent" whereby tree senescence and starvation are induced. Some pathogenic infections require of the host plant to adopt an active and yet suitably altered form of metabolism for the regeneration of the pathogen, at least in the early phases of infection before the products of this altered metabolism become toxic to the host or to the pathogen. Thus, Uromyces phaseoli induced an increase in chlorophyll content around each rust colony in the infected bean leaf, so that active photosynthesis and starch synthesis could take place (Wang, 1961). Susceptible wheat leaves infected with Erysiphe showed increases in their dry weight, total nitrogen, free aminoacids, and protein - aminoacids (Shaw and Colotelo, 1961), due to an altered mode of host metabolism (Shaw and Samborski, 1957). Detachment, or treatment of the leaf bases of normally resistant wheat plants with heat, caused marked increases in the carbohy-

drate and soluble nitrogen content of the leaves, and induced susceptibility to rust infection, which suggested the importance of substrate availability in the maintenance of rust development (Forsyth and Samborski, 1958).

Shoot apices delayed the development of mucus effects in the needles situated close to the apices, in both tree and twig experiments, and the one year old needles were also less susceptible to mucus than the two year old needles. The senescence of pine needle segments or of radish leaf discs, in the dark, was enhanced by mucus but effectively delayed by kinetin; moreover, the senescence - enhancing effect of mucus was overcome by the presence of kinetin. These observations show that juvenility of the tissues, either through the influence of natural plant hormones or from external application of hormones which are known to actively promote synthetic processes and to prevent the destruction of cellular organelles, is important in determining the degree of resistance to mucus. In tobacco plants, the mature leaves were also more susceptible to TMV infection than young leaves; removal of the terminal bud or application of purine-like cytokinins arrested senescence and reduced the number and size of the TMV lesions (Dijkstra and van Rensen, 1968; Kiraly, et al, 1968). The latter suggested that a high rate of RNA and protein synthesis in the leaves had increased their chances of TMV resistance. Further prove for this hypothesis was derived from experiments which involved the use of chloramphenicol (a specific protein inhibitor), or of methods which induced senescence by interfering with normal phloem transport. In detached wheat leaves, application of benzimidazole or kinetin resulted in the maintenance of normal physiological

processes and also induced resistance to rust infection (Wang, et al, 1961). Tobacco leaves infected with Pseudomonas tabaci developed chlorotic halos and contained high levels of free aminoacids and ammonia, but a low level of soluble protein; these changes were arrested and chlorosis completely suppressed, by treating the infected leaves with kinetin (Farkas and Lovrekovich, 1963).

Under experimental conditions, a high dose of mucus injection will kill a tree, just as a tree in the field receiving a "light" Sirex will have a better chance of survival than one receiving a "heavy" Sirex attack. It is suggested that a threshold level of toxicity exists, whereby a minimum quantity of mucus is required to alter the course of metabolic activity in the needle tissues, to one which results in death. This "threshold level" will vary according to the physiological status of the tree prior to the mucus treatment, so that vigorous dominant trees will have a higher threshold level than the weak, damaged or suppressed trees.

The physiological activity of S.noctilio mucus remains stable, after freezing or autoclaving. It is apparently not host - specific. The earlier development of senescence symptoms in trees or twigs, after treatment with autoclaved mucus, is related to the faster uptake of the autoclaved mucus solution when compared with a fresh mucus solution. This is partly due to differences in the relative viscosities of the two solutions, where the fresh mucus loses its very high viscosity after autoclaving. Besides this obvious change in physical character as a result of autoclaving, the question arises as to whether other physical changes occur. What is the nature of this biological entity, and how does it compare with the mucus secretions of other organisms? Some answers to these questions are given in Part II of this thesis.

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PART II

Some physicochemical properties of *Sirex noctilio* mucus

POLYSACCHARIDE - PROTEIN COMPLEXES

In Sirex noctilio the secretion of the mucus gland has been shown to be a complex of carbohydrate and protein (Boros, 1968; Gaut, 1970). These complexes also form important constituents of tissues, body fluids and secretions of most other animals. Of major importance and widespread occurence are the glycoproteins and acid mucopolysaccharides (namely those of hyaluronic acid, the chondroitin sulphates and heparin) although other complexes of limited distribution include the polysaccharide sulphates of molluscs and echinoderms, polysaccharide phosphates of certain invertebrates, chitin present in the exoskeletons of arthropods, and the protein - glycogen complex which forms part of the carbohydrate reserve in some invertebrates.

Definition

Early classifications of polysaccharide - protein complexes were based on the physical properties of the molecules as a whole, and later on the chemical composition of the carbohydrate moieties. Meyer (1938) proposed a classification which was based on the nature of the carbohydrate radical and separated the hexosamine - containing compounds into mucopolysaccharides or glycoproteins. Thus mucopolysaccharides besides containing uronic acid are non-sulphated (e.g., vitreous humor, umbilical cord, synovial fluid, group A hemolytic streptococcus and type I pneumococcus) or sulphated (e.g., chondroitinsulphuric acid, mucoitinsulphuric acid and heparin); neutral mucopolysaccharides of known composition are those that are present in chitin, gastric polysaccharide and bacterial polysaccharide; and glycoproteins which are neutral mucopolysaccharides of unknown composi-

tion, include the α - and β - ovomucoid, serum mucoid, serum glycoid, globulins and pregnancy urine hormone. With increasing chemical knowledge of these compounds, modifications to the original scheme of classification were made by Meyer in 1945 and 1953, and the terms "mucoid" and "mucoprotein" were incorporated. Meyer's classification of the hexosamine - containing compounds in 1945 consisted of three groups, namely the mucopolysaccharides which contained hexosamine as one component; the mucoids or mucoproteins which contained more than 4% hexosamine and were substances which contained a mucopolysaccharide in "firm linkage" with a peptide; and the glycoproteins which were a group of proteins containing less than 4% hexosamine. Mucopolysaccharides were further subdivided into the neutral form (e.g., chitin and type 14 pneumococcus polysaccharide) or the acidic form (e.g., simple: hyaluronic acid; complex and sulphated: hyaluronosulphate from the cornea, chondroitin sulphate and heparin; complex and phosphated: those present in pneumococcus and streptococcus). The mucoids or mucoproteins were either soluble and neutral (e.g., mucoid of the frog oviduct, gonadotropic hormone, blood group A substance and saliva); insoluble (e.g., "ovomucin" or ovomucoid-β , chalaza, residual protein of vitreous humor, and lens capsule); or acidic (e.g., sub-maxillary mucoid). Examples of glycoproteins were ovalbumin, serum albumin, serum globulins and stromatin. In 1953 Meyer defined mucoproteins as salts which resulted from the ionic bonding of mucopolysaccharides and proteins. Mucoids (which contained more than 4% hexosamine) and glycoproteins (which contained less than 4% hexosamine) consisted of covalently linked protein and carbohydrate groups.

Stacey (1946) separated the polysaccharide - protein complexes

into "mucopolysaccharides" and "mucoproteins". The mucopolysaccharides were compounds which were predominantly carbohydrate in nature and contained a low but significant protein content, and mucoproteins were compounds which had a relatively high protein or peptide content and behaved predominantly as proteins in chemical reactions (e.g., ovomucoid, serum mucoproteins, pituitary hormones, and submaxillary mucins). Thus, mucopolysaccharides which contained hexosamine and hexuronic acids were hyaluronic acid, heparin, chondroitin sulphate, mucoitin sulphate from gastric mucin, hyaluronic acid sulphate from the cornea; mucopolysaccharides which contained hexosamines without hexuronic acids were chitin, some bacterial polysaccharides, and blood group substances; mucopolysaccharides which contained hexuronic acids but not hexosamines were the pneumococcus specific polysaccharides (types II, III and VIII) and certain bacterial polysaccharides; and mucopolysaccharides which contained neither hexosamine nor hexuronic acids were the dextrans, levans, mannans, and snail mucin.

Besides Meyer's and Stacey's systems of classifications, various other systems had been proposed by a number of people and the terms "mucopolysaccharide" and "mucoprotein" were defined in different ways by different authors, with resulting confusion as to the true meaning of these terms (Gottschalk, 1966). Jeanloz (1960) based his classification on the type of linkage which existed between carbohydrate and protein and five groups were formed: 1) pure polysaccharides (or glycosaminoglycuronoglycans) e.g., hyaluronic acid, the chondroitin sulphates, and heparin. 2) compounds containing a carbohydrate component attached to a polypeptide component through a "weak" linkage such as salt linkage or hydrogen bonding, e.g., polysaccharide - protein com-

plexes of the group 1 polysaccharides. 3) compounds containing a carbohydrate component attached to a polypeptide component through a "strong" linkage like covalent bonding, e.g., glycoproteins, glycopolypeptides or glycopeptides, depending on the properties of the non - carbohydrate moiety. Groups 4) and 5) refer to glycolipids. The general plan of Jeanloz (1960) was adopted with some modifications by Pearse (1968). In Pearse's scheme, group I consisted of glycans which were further subdivided into the homoglycans (e.g., glycogen, starch, cellulose. dextran and galactan), homopolyaminosaccharides (e.g., chitin), homopolyuronosaccharides (e.g., pectic acid and alginic acid), or heteroglycans (e.g., glycosaminoglycans as found in keratosulphate and glycosaminoglucuronoglycans as found in hyaluronic acid, the chondroitin sulphates and heparin). Group II consisted of polysaccharide - protein complexes formed from the complex of group I glycans with protein. Group III consisted of glycoproteins and glycopeptides, group IV of glycolipids, and group V of glycolipid - protein complexes.

In view of subsequent evidence for the existence of covalent linkage between carbohydrate and peptide groups in the chondroitin sulphate - protein, heparin - protein and chitin - protein complexes, Jeanloz's choice of criterion for the classification of polysaccharide - protein complexes was considered inappropriate (Gottschalk, 1966). Instead, Gottschalk (1966) classified these compounds according to the characteristic structural features of their carbohydrate moieties, and defined glycoproteins as conjugated proteins with a prosthetic group consisting of one or more heterosaccharide. The heterosaccharide group consists of a relatively low number of sugar residues, lacks a serially repeating unit, and is covalently bonded to the polypeptide chain.

Similarly, Hunt (1970) defined glycoprotein as a conjugated protein where the prosthetic group or groups are constituted by covalently bound heterosaccharide units. The polysaccharide unit of glycoproteins consists of usually N-acetylated hexosamines, i.e., either 2-amino-2-deoxy-D-glucose or 2-amino-2-deoxy-D-galactose, or both, which are associated with such monosaccharides as mannose, galactose and fucose, with glucose being occasionally present. Glycoproteins of vertebrate sources are also associated with sialic acids as a prosthetic group, although sialic acids are of less common occurence in the invertebrates.

There is no formal definition for the term "mucopolysaccharide" and Brimacombe and Webber (1964) had applied it to those polysaccharides which contain uronic acid and hexosamine residues. Polysaccharide forms the predominant unit of mucopolysaccharides; it is usually linear, of high molecular weight, and consists of a regularly repeating sequence of neutral sugars and hexosamines or uronic acids. The polysaccharide unit is usually covalently bonded to protein, and the complex may or may not be sulphated. Examples of mucopolysaccharides are hyaluronic acid, the chondroitin sulphates and heparin.

In view of the wide usage of different terminologies, such as given in this survey, I propose to adopt the more "popular" terms in this thesis i.e., "glycoprotein" and "mucopolysaccharide" as defined by Gottschalk (1966), Brimacombe and Webber (1964), and Hunt (1970).

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Histochemical detection

Polysaccharide - protein complexes in biological material are readily detected by means of histochemical stains, in combination with certain chemical reactions for the purpose of distinguishing between closely related groups of compounds. The diagnostic chemical reactions used in the determination of major classes of compounds, in routine laboratory histochemistry, include the dinitro-fluoro-benzene (abbreviated DNFB) or tetra-azotised anisidine methods for protein, toluidine blue (at pH 6, 4.2 or 1) for acidic groups, periodic acid - Schiff test (abbreviated P.A.S.) for carbohydrates, and Sudan black for lipids (Chayen, et al, 1969). Carbohydrates stain red or purple - red with P.A.S., and the P.A.S. reactivity is lost after benzoylation. Simple carbohydrate polymers like glycogen and starch lose the P.A.S. colour on rinsing with water or dilute acids, but confirmation of these carbohydrates may be achieved with tests using iodine (glycogen stains red and starch stains blue or purplish - blue) and Best's carmine, together with digestions with α- and β- amylase. Although Best's carmine was held to be an empirical stain for glycogen (Gomori, 1952), recent indications are that the reaction is only sensitive towards branched glycogen polymers, and not to the long chain polymers. Acidic conjugated proteins, specifically of protein - acidic polysaccharide complexes, stain red - purple with DNFB, red or brown - red with tetra-azotised dianisidine, red or purple - red with P.A.S. (the P.A.S. reaction is sensitive to benzoylation but is not affected by rinsing with water or dilute acids), and blue or red with toluidine blue at pH 4.2. Both nucleic acids and acidic polysaccharides stain with toluidine blue at pH 4.2 or less, and persistence of staining at pH 1 is due to the pre-

sence of phosphate or sulphate groups, whereas abolition of staining at pH values of less than 4 indicates the presence of such weak acidic groups as carboxyl. Azure A may be used in place of toluidine blue.

In the early days of histological staining, it was customary to determine the degree of basophilia in acidic mucopolysaccharides, as a means of qualitative analysis. Haematein and carmine were commonly used, and later replaced with methylene blue. By measuring the lowest pH value at which the compound could react with methylene blue, the "methylene blue extinction" was obtained. The degree of basophilia is inversely related to the methylene blue extinction. Other dyes have since been used for this purpose, and Spicer (1960) reported that Azure A (a thionin dye) in HCl - phosphate or phosphate - citrate buffers, at a range of pH 1.5 to 4.0, could be used to distinguish between the carboxylic and sulphate groups of acid mucopolysaccharides. Thus, sulphate groups which ionised at pH 1.5 were able to combine with Azure A, but not the carboxylic groups which were unionised at this pH. However, theoretical and practical objections were raised with regards to the use of such chemicals for the specific identification and distinction of carboxyl and sulphate groups (Pearse, 1968).

The histochemical handbook of Chayen, et al (1969) employs alcian blue (a copper - phthalocyanin dye) and Hale's colloidal iron as diagnostic stains for acid mucopolysaccharides or "mucins". The colloidal iron method depends on the adsorption of colloidal iron by tissue components (probably acidic groups), and subsequent staining of iron by the Prussian blue method. There is little reason to believe that the colloidal iron method is intrinsically specific for any chemical grouping, but in Pearse's experience (Pearse, 1968) the reaction is useful

and extensively applied as a histological stain for mucopolysaccharides, acidic polysaccharides, or for some types of "mucins".

Alcian blue was first introduced by Steedman (1950) as a histochemical stain. It is considered extremely useful and valuable for "mucins" and acid mucopolysaccharides which stain blue (Chayen, et al, 1969); and in an acidic medium of pH 2.7 to 3.0 only acidic "mucosubstances" stain blue - green (Sumner and Sumner, 1969). The specificity of alcian blue staining had been the subject of much debate, until the work of Spicer (1960) who showed that at pH 2, alcian blue reacted mainly with the uronic acid groups of the mucosubstances. However at pH 1, alcian blue staining was specific for sulphated mucopolysaccharides.

Several hypotheses had been formulated on the nature of alcian blue bonding with acid mucopolysaccharides. That the staining of uronic acids was attributed to the formation of amide bonds (Spicer, 1960) was discounted by Pearse (1968) in favour of Stoward's hypothesis (Stoward, 1963, as quoted by Pearse) which suggested the formation of hydrogen bonding between the unionised carboxyl to copper and phthalocyanin nitrogen to form a stable 6 - membered structure. Scott, et al (1964) postulated that alcian blue combines with polyanionic substances by means of salt linkages, since prior treatment of tissue sections with cetylpyridinium chloride had resulted in the complete inhibition of their reaction with alcian blue. A series of papers by Scott and his associates (Quintarelli, et al, 1964; Scott, et al, 1964; Quintarelli and Dellovo, 1965) culminated in the establishment of the "critical electrolyte concentration" method, in conjunction with alcian blue staining, as a means of differentiating acidic mucosubstances in tissues. In the presence of low electrolyte concentrations (e.g., be-

low 0.3M $MgCl_2$), sulphated mucins and glucosaminoglucuronoglycans containing carboxyl groups will bind with alcian blue, but at higher electrolyte concentrations (e.g., above 0.8M $MgCl_2$) only sulphated mucosubstances will bind with alcian blue (Scott and Dorling, 1965).

A variety of chemicals have been used for the detection of acid mucopolysaccharides. These are generally basic in nature and belong to the following classes: azo, thiazin, oxazin, azin, triphenylmethane, and acridine. Jacques, et al (1947) noted the following changes to the colour of the dye solutions after the addition of heparin: Bismarck brown (colour changed from orange to yellow), azure A (blue to red), brilliant cresyl blue (blue to purple), cresyl violet (blue to purple), bromocresol blue (blue to purple), Nile blue sulphate (greenish blue to reddish blue), neutral red (red to orange, at pH 6 - 7), basic fuchsin (a shift in the red colour), pyronin (the pink solution lost its fluorescence) and acriflavin (the solution lost its fluorescence). These are metachromatic reactions and are typical of highly acidic mucopolysaccharides like heparin. By combining salt elution with acridine orange staining, Saunders (1964) was able to differentiate between the three types of acid mucopolysaccharides. It was found that in the absence of NaCl, all of the three acid mucopolysaccharides had stained fluorescent red, but in the presence of increasing concentrations of NaCl the weaker acid mucopolysaccharides tended to lose their staining. Thus, hyaluronic acid stained with acridine orange at neutral pH in the absence of NaCl; both chondroitin sulphate and heparin stained at pH 3.2 in the presence of 0.3M NaCl; only heparin stained at pH 3.2 in the presence of 0.6M NaCl.

Chitin, a neutral mucopolysaccharide, also reacts with P.A.S.,

stains orthochromatically with toluidine blue, and reacts with Hale's colloidal iron and alcian blue. Runham (1961) found that the intensity of staining with P.A.S. was reduced after deamination and negated after acetylation (acetylation results in the blocking of hydroxyl groups on the sugar molecules), but restored after deacetylation. As the P.A.S. reaction was not affected by diastase, hyaluronidase or pectinase, Runham (1961) concluded that the reactivity of chitin towards P.A.S. was due to carbohydrates other than glycogen, hyaluronic acid, some chondroitin sulphates, polygalacturonic acid or galactogen. Reactions with alcian blue and Hale's colloidal iron were also blocked by methylation and acetylation.

Histochemistry is a science of great antiquity which in the view of Pearse (1968) has progressed without interruption. Inspite of continual alterations and improvements to original techniques, the aims and principles of this science have been preserved throughout the years. Histochemistry provides information on the distribution of various types of compounds present *in situ*. It cannot be relied upon as the sole basis on which identifications of chemical compounds are made.

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Some preparative and analytical procedures which aid in the characterization of glycoproteins and mucopolysaccharides

Prior to studies on the chemical and physical properties of polysaccharide - protein complexes in biological material, an initial procedure involving the elimination of associated lipids, proteins or carbohydrates may have to be performed before the isolation and purification of the complexes. Lipids are dissolved in the appropriate solvents; simple carbohydrates are digested with enzymes; and proteins are removed by precipitation or dialysis following digestion with proteolytic enzymes. Removal of proteins may be achieved with alkali treatment, but there is a possible degradative effect on the polysaccharide moiety of the polysaccharide - protein complex as a result of this treatment.

Most of the well - founded techniques of isolation and purification of glycoproteins and acid mucopolysaccharides have been developed and applied on vertebrate material. These include gel filtration, ion - exchange chromatography, electrophoresis, density gradient centrifugation, and precipitation with aliphatic ammonium salts. Details of these procedures and of their applications are to be found in Gottschalk (1966), "Methods in Enzymology" (1966 and 1972), and "Methods of Biochemical Analysis".

A variety of gel filtration media and ion - exchange resins have been manufactured. Some have proved to be more discriminating than others, in separating individual polysaccharide - protein complexes. Glycoproteins have been satisfactorily isolated by filtration through columns of Sephadex, Biogels and cellulose, and eluted from such cationic exchangers as Dowex - 50, carboxymethyl - cellulose, and DEAE - cellulose. To a certain extent, anionic exchangers like DEAE - Sephadex

and Dowex - 1 have also been used for glycoproteins. Individual acid mucopolysaccharides are readily isolated by means of selective salt elution from anionic exchangers (e.g., Dowex - 1, - 2, - 3, Amberlite CG - 45, Duolite A - 4, Ecteola - cellulose, and DEAE - Sephadex). Clear cut separations of hyaluronic acid, heparitin sulphate, chondroitin sulphate, and heparin, have been achieved with DEAE - Sephadex and a stepwise - increase in NaCl concentration (Schmidt, 1962).

The use of aliphatic ammonium salts in the extraction and purification of acidic polysaccharides was reviewed by Scott (1960). Acid mucopolysaccharides form complexes with quaternary ammonium salts like cetylpyridinium chloride, cetyl trimethylammonium bromide and cetyl dimethylbenzylammonium chloride, in a stoichiometric manner. The different complexes precipitate from solution at "critical salt concentrations", and the value of critical salt concentration for each acid mucopolysaccharide is directly related to the charge density on the molecule itself. Complete isolation of individual acid mucopolysaccharides from a mixed solution was achieved with cetylpyridinium chloride and salt solutions; this method was also successfully applied to the isolation of heparin from rat skins (Schiller, et al, 1961).

Electrophoresis as a means of isolating individual polysaccharide - protein complexes has not been extensively applied. However, hyaluronic acid was successfully separated from chondroitin sulphate B by electrophoresis on a slab of Celite analytical filter aid which had been moistened with NaCl and phosphate buffer at pH 7 (Schiller, et al, 1954).

Having isolated the glycoprotein or mucopolysaccharide, a proof of homogeneity is then carried out. These methods involve gel filtration, ultracentrifugation, electrophoresis (moving boundary or on sup-

porting media), column or paper chromatography, and immunology, although biological tests for homogeneity (e.g., enzymatic activity) may also be used when practicable. Brimacombe and Webber (1964) considered that if the results from any two of the aforementioned tests indicate the presence of a single molecular species in a certain preparation, then the preparation can be assumed to be homogeneous. The term "homogeneous" is not rigorously definable (Gottschalk, 1966), and is generally used to indicate a pure preparation from which all extraneous matter has been removed.

Characterization involves the identification of constituent compounds, and their quantitation. Sulphate is determined turbidimetrically as a consequence of its precipitation with Ba^{2+} or spectrophotometrically with benzidine or chloranilate. Hexuronic acids are highly susceptible to acidic hydrolysis and are easily decarboxylated, but the amount of CO_2 which is released from the acid hydrolysis gives a measure of the amount of hexuronic acids present. Spectrophotometric methods available for the measurement of hexuronic acids involve the use of orcinol, resorcinol, or carbazole reagents. Hexoses are measured by a number of methods, of which the anthrone, phenol - sulphuric acid, and the cysteineHCl - sulphuric acid methods are most frequently used. Hexosamines are measured by the Elson - Morgan reaction for 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose, or by the Morgan - Elson reaction for N-acyl-hexosamines, e.g., 2-acetamido-2-deoxy-hexoses. Sialic acids, a prosthetic group of most vertebrate and of certain invertebrate glycoproteins, are measured by the resorcinol, Ehrlich or thiobarbituric acid methods.

Thus, several procedures are available for the determination of

any one class of sugars, and procedures have also been devised for the measurement of specific types of sugars. The choice of any one particular method for the assay of any one class of sugars largely depends on the presence of certain associated compounds, and on the degree of interference which may be exerted by those compounds on the assay procedure. It is not practicable here, to detail and evaluate all of the methods which are available for the assay of sugars and sulphate, but details are available in the reference texts, e.g., "Methods in Enzymology" and "Methods of Biochemical Analysis".

Tentative identifications of constituent sugars by chromatography on paper or silica, are based on their rates of mobility in specified solvent systems and on the colour of their products following chemical reactions with "vizualization reagents", in relation to authentic sugar samples. Although much attention is focused on the polysaccharide moiety of glycoproteins and acid mucopolysaccharides, useful information may be derived from determination of the aminoacid composition.

A complete identification of the complex involves additional study on the structure and configurational properties of all oligosaccharides which have been released by controlled acidic or enzymatic hydrolysis, or following periodate oxidation or methylation, and the preparation of crystalline derivatives from which the melting point, infrared spectrum and optical rotation are determined. These procedures are dealt with in "Comprehensive Biochemistry" (1963), and by Bailey and Pridham (1962).

It is beyond the scope of the present investigations on Sirex mucus to completely resolve the chemical identity of the mucus. However, information regarding some aspects of its physical and chemical proper-

ties would allow a tentative classification of the polysaccharide - protein complex(es) present.

The isolation and characterization of some invertebrate polysaccharide - protein complexes

Although conditions for the isolation and characterization of glycoproteins and acid mucopolysaccharides from vertebrate sources do not strictly apply to material from invertebrate sources, they have nevertheless been successfully adapted for invertebrate studies. A survey of some preparative procedures for invertebrates is given below.

From the egg jelly coat of the starfish Asterias amurensis, a pure preparation of mucopolysaccharide was obtained, after the elimination of extraneous matter with 90% phenol, and precipitation of the mucopolysaccharide with protamine. The mucopolysaccharide appeared as a single component on ultracentrifugation and electrophoresis, and contained large amounts of galactose, fucose and sulphate, less of hexosamine and none of uronic acids or sialic acids (Muramatsu, 1965).

Mucin from the hypobranchial gland of Buccinum undatum (Mollusca) was isolated with 0.5M saline at 4°C, and found to be composed of a glycoprotein and glucan sulphate. These two major components were readily separated from each other with phenol treatment, analytical ultracentrifugation or electrophoresis. The glycoprotein consisted of glucose, galactose, mannose, fucose, glucosamine and galactosamine; the glucan sulphate consisted of glucose and sulphate in the respective proportions of 54% and 30% (Hunt and Jevons, 1963, 1965).

Sephadex G75 was employed for the fractionation of papain digests of mucin from the hypobranchial gland of Neptunea antiqua (Doyle, 1964).

The Molisch - positive fractions were metachromatic at pH 2 and appeared as a single zone on paper electrophoresis, with a rate of mobility being half that of standard mammalian chondroitin sulphuric acid. It consisted of glucosamine, galactosamine, glucose and fucose, but uronic acid was absent. The mantle mucin of Pecten maximus (Mollusca) was digested with papain and eluted from DEAE - Sephadex into three peaks, with 0.1M NaCl, 0.5M NaCl, and 4M NaCl, respectively (Doyle, 1967). Differences were detected in the monosaccharide composition of these three fractions, but only the last fraction was metachromatic at pH 2 and contained glucose with traces of a pentose.

A pronase digest of the Cuverian tubules of the sea cucumber, Holothuria forskali, was eluted from Sephadex G-50 with pyridine - acetate buffer, 0.1M, pH 6, into three glycopeptide fractions (Isumera, et al, 1973). Further purification was achieved by filtration of the glycopeptides through columns of Sephadex - G100, AG - Dowex 50W (X2), Dowex 1 (X2), or Sephadex G-25. The first glycopeptide was found to be a sulphated polyfucose containing galactosamine, a uronic acid and a neuraminic acid derivative; the second glycopeptide consisted of a neuraminic acid derivative, galactose, mannose and glucosamine, and was similar to the group of globular glycoproteins; the third glycopeptide fraction was made up of two different glycopeptides.

Using Biogels (P series) as molecular seives, the separation of glycogen from other sulphated heteropolysaccharides was achieved, from acetone powders of nine different species of marine invertebrates (Ovodov, et al, 1969). Similarly, the initial separation of sialopolysaccharide - protein complexes from other glycoproteins present in the egg jelly coats of the sea urchin, Pseudocentrotus depressus, was

achieved through the use of a column of Biogel P100 (Hotta, et al, 1970). After further filtration on Biogel P200, a pure sialic acid - rich protein fraction containing fucose, glucose, mannose and a trace of galactose was obtained. A disaccharide consisting of fucose and sialic acid, first isolated from the egg jelly coats of sea urchins by preparative thin layer chromatography, was purified by filtration on Biogel P2 (100 - 200 mesh) (Hotta and Kurokawa, 1973).

Mucopolysaccharides from the slime of the snail, Otella lactea, were precipitated with cetylpyridinium chloride and chromatographed on DEAE - cellulose. Glucosamine, galacturonic acid, glucose and fucose, together with one unidentified sugar, were identified from this preparation (Pancake and Karnovsky, 1967). Got and Marnay (1968) isolated two glycoproteins, each with enzymatic activities, from the digestive juices of the snail (Helix pomatia) by means of filtration on Sephadex G-200, precipitation with 2.8M $(NH_4)_2SO_4$, and chromatography on ion - exchangers. The first enzyme was chromatographed on carboxymethylcellulose and the second enzyme was chromatographed on DEAE - Sephadex. Although having a similar carbohydrate content and composition (i.e., galactose, mannose, glucose, fucose and glucosamine), and similar isoelectric points, the two enzymes had different sedimentation coefficients and different rates of migration on starch gel electrophoresis.

Mucopolysaccharides isolated from defatted and proteolytically - digested midgut tissues of the greater wax moth, Galleria mellonella, appeared to be similar to mammalian hyaluronic acid on ECTEOLA - cellulose chromatography (Estes and Faust, 1964).

Some of the procedures involved in the isolation of polysaccharide - protein complexes consisted of elimination of extraneous material

by precipitation or gel filtration, although ion - exchange chromatography appears to be gaining in importance. A variety of polysaccharide - protein complexes which have been isolated from the tissues and body secretions of various invertebrates have been shown to consist of mixtures of glycoproteins and acid mucopolysaccharides. In the following chapter, a survey is made on the polysaccharide - protein complexes of insects.

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Polysaccharide - protein complexes of insects

The detection of polysaccharide - protein complexes in insects, at first was based mainly on histochemical reactions. Using the Gomori test for glycogen, Bismarck brown test for water - labile mucoproteins and toluidine blue for detection of metachromasia, Day (1949) was able to demonstrate the presence of "mucoid substances" in the cuticle, chitinous intima, striated border of the midgut epithelium, striated border of some Malpighian tubules, fat body, peritrophic membrane, connective tissues, and salivary glands, in some of the nine different species of insects which he examined. The microscopic organic fibrils present in the dwelling tubes of machaerotid larvae (Machaerotidae: Homoptera) stained intensely with alcian blue. They were gamma-metachromatic with toluidine blue, positive in the Hale's test and with mucicarmine, but did not stain with the P.A.S. or with protein reagents. Marshall (1968) suggested that acid mucopolysaccharides were present, and hydrolysates of these dwelling tube fibrils contained glucuronic acid, glucosamine and glucose.

The connective tissue sheath which separates the haemocoele from the rest of the internal organs of the body, of three species of sucking lice, was reactive with P.A.S. even after treatment with diastase. It was either colourless or stained a light blue with toluidine blue, it had a methylene blue extinction in the range of pH 5.2 - 6.2, and these reactions were not affected by the presence of boiling water but were completely dispersed by treatments with alkali or pepsin. The results of these investigations led Pipa and Cook (1958) to suggest the presence of a neutral mucopolysaccharide, mucoprotein or glycoprotein.

Connective tissue associated with the central nervous system of

the wax moth Galleria mellonella is made up of the neural lamella, sheath cells and dorsal mass of connective tissues. Ashhurst and Richards (1964a) interpreted the histochemical reactions of the neural lamella as being those of neutral mucopolysaccharides in the larva, with acid mucopolysaccharides also being present in the adult. The cytoplasm of the sheath cells was thought to contain neutral mucopolysaccharides, and the dorsal mass of connective tissues appeared to consist of neutral and acid mucopolysaccharides. In the cockroach Periplaneta americana, the neural lamella was strongly P.A.S. positive and the reaction was not affected by diastase, hyaluronidase or saliva. It was beta-metachromatic with toluidine blue, and had a methylene blue extinction above 5, suggesting the presence of neutral mucopolysaccharides. The sheath cells contained glycogen, phospholipids and cerebrosides, protein and nucleic acids, and the presence of hydroxyproline in the protein of the sheath cells suggested that it was of a collagen type (Ashhurst, 1961).

In the wax moth, Galleria mellonella, the adipohemocytes of the larval blood cells contained histochemically identified neutral mucopolysaccharides or mucoprotein, whilst the spherules contained acid mucopolysaccharides (Ashhurst and Richards, 1964b). Two glycoprotein fractions were isolated from the plasma of P.americana. These were composed of glucosamine, galactosamine, glucose, mannose, galactose, arabinose and xylose (Lipke, et al, 1965). Mannose was the principal monosaccharide, and glucosamine was the principal hexosamine, in the plasma in between moults. On paper electrophoresis the cockroach plasma was separated into five fractions, of which fractions II and IV contained carbohydrates and phospholipids (Siakotos, 1960a).

The maxillary gland of the milkweed bug Oncopeltus fasciatus, is composed of large peripheral cells within which glandular secretions are formed. Smaller centrally located cells surround a system of inter- and intracellular chitinous ductules. These ducts open separately into the glandular reservoir and terminate in the peripheral cells. Within the peripheral cells, the ducts are modified to form a bulb and terminal duct. A primary vesicle surrounds the terminal duct. High alkaline phosphatase activities were located in the primary vesicle and terminal duct. Secretions of the peripheral cells were proteinaceous, non-metachromatic, and markedly acidophilic, which suggested the presence of mucoprotein or neutral mucopolysaccharide. However as its reaction with P.A.S. was not affected by diastase, acid mucopolysaccharides were thought to be also present (Linder, 1956).

Mucoprotein or glycoprotein and neutral mucopolysaccharide appeared to be present in the anterior lobe, lipoprotein in the lateral lobe and mucoprotein in the posterior lobe, of the trilobed principal salivary gland of Oncopeltus fasciatus (Dallas) (Salkeld, 1960). Thus, basophilic granules and globules of the anterior lobe were strongly beta-metachromatic (i.e., violet) with toluidine blue, and metachromasia was not affected by hyaluronidase or ribonuclease. They also stained blue-green with alcian blue, deep-brown in Bismarck brown, brilliant red in mucicarmine, and had a methylene blue extinction above pH 4; but were negative with the P.A.S. reagent. The acidophilic fine grains of the lateral lobe were beta-metachromatic with toluidine blue (reaction was unaffected with hyaluronidase or ribonuclease) and had a methylene blue extinction above pH 5.3. The reaction of these fine grains with P.A.S. was not affected by saliva, diastase or pyridine but was sensitive to

acetylation. On the other hand, the homogeneous material of the posterior lobe was purple in eosin - methylene blue, orthochromatic (i.e., blue-green) in toluidine blue, and reacted with methylene blue above pH 4.6. Its reaction with alcian blue and P.A.S. was faint. The P.A.S. reaction was also blocked by acetylation and restored with deacetylation.

A gummy substance secreted by the larvae of Chironomus thummi, contained protein which was separated into fourteen bands by electrophoresis on cellulose acetate strips. Ten of these bands were metachromatic with toluidine blue but did not react with P.A.S. reagent probably due to the presence of low concentrations. Kato, et al (1963) suggested that mucopolysaccharides may be present. Differences in the intracellular distribution pattern of mucopolysaccharides in the salivary glands of Smittia sp. (Chironomidae) and Bradysia mycorum Frey. (Sciaridae) were demonstrated by histochemical means. A mixture of neutral mucopolysaccharide and sulphated and unsulphated mucopolysaccharides were detected in the salivary glands of these two species (Kato and Sirlin, 1963). The salivary gland secretion of the pupa of Drosophila virilis serves as an adhesive for attachment of the pupa to the substratum. An aqueous solution of this secretion, with a pH of 9, reacted intensely with the P.A.S. reagent and contained "free" protein (amino acids), reducing sugars and aminosugars (Perkowska, 1963). These were identified chromatographically as alanine, valine and three other aminoacids, glucose, galactose and mannose, and glucosamine. Immunoelectrophoretic studies suggested a non-homogeneous mixture of muco-protein which was also metachromatic with toluidine blue and stained blue with bromophenol.

Attached to the midgut of the silkworm, Bombyx mori, is a soft,

thin membrane called the peritrophic membrane. This membrane is constantly being renewed as it wraps itself around the food ingested by the silkworm, and is discharged along with the faeces. Chitin, protein and a polysaccharide consisting of equimolar amounts of glucuronic acid and glucosamine residues were present in the peritrophic membrane (Nisizawa, et al, 1963). On the basis of evolution of CO_2, presumed to be derived from glucuronic acid, the authors suggested that hyaluronic acid may be present, comprising about 15% of the peritrophic membrane. A mucopolysaccharide isolated from the midgut of Galleria mellonella was identified as hyaluronic acid. Its elution from ECTEOLA - cellulose was similar to that of mammalian hyaluronic acid (Estes and Faust, 1964).

Histochemically similar mucopolysaccharides appeared to be present in the Malpighian tubules, hindgut and spittle of the cercopid larvae (Homoptera). Acid hydrolysis of the spittle and Malpighian tubule secretion produced monosaccharides which were tentatively identified as glucuronic acid, glucosamine, glucose and mannose. Marshall (1966) suggested that a Malpighian tubule mucopolysaccharide was periodically extruded and passed down the hindgut to form a component of the spittle, possibly functioning as a surface-tension depressant.

The dermal glands of the nymphs of Rhodnius prolixus produce a clear hyaline secretion which gave histochemical reactions typical of mucins (Baldwin and Salthouse, 1959). This secretion was positive in alcian - mucicarmine, metachromatic in toluidine blue, and positive in alcian blue. However loss of staining with alcian blue after treatment of the secretion with bovine testicular hyaluronidase or with liver B-glucuronidase led the authors to suggest the presence of hyaluronic acid in the mucin.

Moulting fluid from the fourth larva and from the pupal moult of the silkworm Bombyx mori, contained high activities of protein - and chitin - digesting enzymes, together with the products of protein and chitin hydrolysis. Also present were seventeen aminoacids (i.e., arg, α-ala, asp, aspn, cysteine, phe-ala, gly, glu, his, leu, lys, met, pro, ser, threo, tyro, and val), N-acetylglucosamine and glucosamine (Zielinska and Laskowska, 1958). Fibroin prepared from newly spun cocoons of the silkworm was subjected to sequential digestion with trypsin and pronase, and found to consist of two glycopeptide units of which their molecular weights were estimated to be 1600 and 1100, respectively, from Sephadex G-50 fractionations. One of these glycopeptides was made up of two residues of glucosamine and five residues of mannose, whilst the other glycopeptide of a lower concentration, was made up of two residues of glucosamine and three residues of mannose. The molecular weight of fibroin was calculated to be 4×10^5, on the basis of carbohydrate compositions of the entire fibroin and of each of the glycopeptides (Sinohara, et al, 1971).

Diapause eggs of the grasshopper, Melanopus differentialis, are surrounded by four distinct layers which comprise the exo- and endochorion, and the inner and outer cuticle. Using histochemical means, Jahn (1935) reported that the outermost thick white fibrous cuticular layer was composed wholly of chitin, the thin yellow cuticle just within it did not contain chitin or protein, and the two chorion layers were composed of proteins with differing isoelectric points but did not contain chitin.

The eggshell of Nepa cinerea (Nepidae: Hemiptera) is covered with a hygroscopic viscous material which behaved as an acid mucopoly-

saccharide in histochemical stains: it stained strongly with P.A.S., it was metachromatic (i.e., red) with toluidine blue, and reacted faintly with Millon's reagent (Hinton, 1961). This viscous material serves as an adhesive and is believed to have been secreted by the colleterial glands in Drosophila melanogaster. Fourteen aminoacids in appreciable quantities and trace amounts of one or two other aminoacids were detected by paper chromatography of the acid hydrolysates of the eggshell and its adhesive (Wilson, 1960). On ion - exchange chromatography, using Dowex 50, three peaks were obtained which reacted with the Elson Morgan reagent; the second peak was eluted in the position of authentic glucosamine and constituted 0.6% by weight of both the eggshell and its adhesive (Wilson, 1960). Further histochemical studies on the eggshell adhesive of D.melanogaster by Riley and Forgash (1967) suggested that the adhesive material was secreted by paired accessory glands. Both the adhesive material and the accessory glands were not susceptible to saliva or diastase in their reactions with the P.A.S. reagent; they stained magenta in P.A.S. - alcian blue, and had a methylene blue extinction above pH 4 but did not react with alcian blue and were not metachromatic in toluidine blue. On acid hydrolysis the eggshell adhesive was found to contain eight to ten aminoacids together with glucosamine. The authors suggested the presence of mucoprotein.

The parasitoid Mesoleius tenthredinis lays its eggs in the host, larch sawfly Pristiphora erichsonii. Occasionally, the eggs become encapsulated by the accumulation of blood cells from the host insect. During the formation of the egg capsule, the blood cells become flattened, fibrous, enucleate, and eventually form a mass of concentric sheaths

of non cellular material. The definitive capsule becomes both cellular (outer region) and non cellular (inner region). Histochemical tests indicated the presence of mucopolysaccharides in the egg capsule (Bronskill, 1960); thus the outer sheaths of the capsule were eosinophilic, positive with P.A.S. before and after treatment with diastase, and stained greenish blue with toluidine blue at pH 5.0. The reaction with toluidine blue was not affected by ribonuclease or hyaluronidase treatments. The inner sheaths of the capsule were basophilic, stained with P.A.S. even after treatment with malt diastase, and was beta-metachromatic with toluidine blue at pH 5.0. Ribonuclease or hyaluronidase had no effect on the toluidine blue reaction.

McFarlane (1962) investigated the action of proteolytic enzymes like pepsin, "purified trypsin" and crystalline trypsin on the eggshell of Acheta domesticus (L) (Orthoptera: Gryllidae). Pepsin effectively digested the vitelline membrane of water - swollen eggs after 12 hours of treatment, but had no effect on the newly laid eggs even after 24 hours of treatment. "Purified trypsin" had no effect on newly laid eggs but partially digested the water - swollen eggs. Crystalline trypsin had no effect on newly laid eggs, but attacked swollen eggs after they had received prior treatment with pepsin. The apparent resistance of the eggs to proteolytic digestion may be attributed to a layer of mucoprotein on the surface of the eggs (McFarlane, 1962).

The ovariole of the cockroach, Periplaneta americana, is covered by an inner layer of non cellular connective tissue, the tunica propria and a cellular sheath, the external ovariole sheath. The tunica has a primary supporting function, it aids in ovulation and may serve as a dialytic membrane. It reacted intensely with the P.A.S. reagent (amy-

lase had no effect on the P.A.S. reaction) and with lead tetra-acetate - Schiff, but it did not stain in alcian blue or Bismarck brown. Bonhag and Arnold (1961) suggested that the P.A.S. reactivity of the tunica propria may be due to mucopolysaccharides or mucoproteins. The external ovariole sheath is composed of three layers, the sheath tissue proper, mycetocytes and tracheal tissue. The P.A.S. reactivity of the external ovariole sheath cells was susceptible to amylase, thus suggesting the presence of glycogen. This was then confirmed with Best's carmine and the lead tetra-acetate - Schiff reactions. However, as amylase treatment did not totally remove all of the P.A.S. stain, and as the external ovariole sheath cells were positive in alcian blue, a small amount of acid mucopolysaccharides appeared to be present also. The mycetocytes of adult cockroaches also contained glycogen.

In Cynips folii (Cynipoidea: Hymenoptera), the venom gland consists of a single tube with a single epithelial layer and a permanent regular central canal which opens into a sac like receptacle. Protein, non - specific esterases and alkaline phosphatases were present in the epithelial lining of the venomous gland, whilst phospholipids, lipase and acid phosphatase were present in the gland cells (Krainska, 1966a, 1968). There was a slight reaction with P.A.S., and the lack of staining with alcian blue led Krainska (1966a) to suggest on the possible presence of some neutral polysaccharide but absence of acid mucopolysaccharides in the venom gland. The venom of the honey bee, Apis mellifera (Hymenoptera) consists of a complex mixture of carbohydrates, lipids, free aminoacids, peptides, proteins and enzymes (O'Connor, et al, 1967). Its moisture content was estimated at 88% and protein constituted 60% of the dry matter. The free aminoacids were made up of ala,

arg, cystine, glu, his, pro, and thirteen others in trace amounts. The sugars consisted of glucose and fructose. The oviduct gland of C.folii appeared to consist of mucopolysaccharide - protein globules and acid mucopolysaccharide - phospholipid - protein globules, on the basis of histochemical staining. Krainska (1966b) suggested that the mucopolysaccharides present in the globules were hyaluronic acid and ester sulphated polysaccharides, from a series of tests which included the use of such treatments as methylation, saponification and acetylation.

Mucus secreted by the mucus gland (an accessory gland) of the female Sirex noctilio was provisionally identified as an acid mucopolysaccharide - protein complex by Boros (1968) because it reacted with the Millon reagent, stained a dense purple - pink with P.A.S. (reaction with P.A.S. was not affected by acetylation), it was blue - green in alcian blue, and it had a methylene blue extinction of pH 2.6. Its reaction with toluidine blue was beta-chromatic (i.e., blue) and appeared to be susceptible to hyaluronidase. Following acid hydrolysis and chromatography on paper, eight ninhydrin - reactive bands, five reducing sugar bands, one band of hexosamine and three bands of hexuronic acids were detected. Moreover, reaction of the mucus hydrolysate with $BaCl_2$ resulted in the formation of a fine white precipitate, thus indicating the presence of sulphate. However, Gaut (1970) reported that the mucus behaved as a neutral compound on electrophoresis, it was not metachromatic with Azure A, and it was not precipitated with quaternary ammonium salts. The acid hydrolysates of mucus contained no detectable sulphate and were not similar to authentic hyaluronic acid, chondroitin sulphate and heparin on paper chromatography.

References for polysaccharide - protein complexes in insects

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EXPERIMENTAL WORK

Some physical and chemical properties of Sirex noctilio mucus

Introduction

Invertebrates are essentially "soft" organisms, except for those with tough exoskeletons, and are of abundance in both aquatic and terrestrial environments. They contain a greater variety of polysaccharide - protein complexes than would be present in the vertebrates. Whilst a sharp distinction occurs between glycoproteins and acid mucopolysaccharides in the vertebrates, the distinction is so vague in the invertebrates that uronic acids and ester sulphates are often present in invertebrate glycoproteins, whilst invertebrate mucopolysaccharides may be free of hexosamines and uronic acids (Hunt, 1970).

In insects, hyaluronic acid was characterised in the dermal glands of nymphs of Rhodnius prolixus (Baldwin and Salthouse, 1959), peritrophic membrane of Bombyx mori (Nisizawa, et al, 1963), and midgut of Galleria mellonella (Estes and Faust, 1964). Unspecified mucopolysaccharides were located in the maxillary glands of Oncopeltus fasciatus (Linder, 1956) and dwelling tubes of the machaerotid larvae (Marshall, 1968), whilst a mixture of neutral, sulphated and unsulphated mucopolysaccharides were present in the salivary glands of Smittia sp. and Bradysia mycorum (Kato and Sirlin, 1963). Ester sulphated polysaccharides were also detected in the oviduct gland of Cynips folii (Krainska, 1966).

An acid mucopolysaccharide - protein complex was provisionally identified in Sirex noctilio mucus (Boros, 1968), after a series of histochemical reactions and some paper chromatographic analyses of the mucus hydrolysates. Sulphate was also detected in the acid hydrolysate.

Gaut (1970) reported that Sirex noctilio mucus had a rate of electrophoretic mobility (on agar gel) which was similar to that of dextran, it contained no detectable sulphate, it was not precipitated with quaternary ammonium salts, and its acid hydrolysate was not comparable with the acid hydrolysates of authentic (vertebrate) hyaluronic acid, chondroitin sulphate or heparin on paper chromatography. As a result Boros' identification of S.noctilio mucus was refuted, and instead, Gaut (1970) suggested that S.noctilio mucus is probably a neutral polysaccharide which contains fructose.

The apparently conflicting conclusions on the nature of S.noctilio mucus, drawn by Boros and Gaut, suggest that the mucus is either a unique entity which will not necessarily fall into the class "glycoprotein" or "acid mucopolysaccharide" as defined for the vertebrates, or that it consists of a mixture of complexes which possess the properties of both neutral and acid polysaccharides. Thus, depending on the techniques employed for the characterization of the complex, certain features will be accentuated, resulting in its being identified as one or the other class of compounds. With some exceptions, the procedures employed by Boros (1968) and Gaut (1970) were different.

A pre-requisite for the eventual identification of any compound is the use of homogeneous material, i.e., material from which extraneous matter have been excluded. Unfortunately, few if any steps were undertaken by these two workers in establishing the homogeneity of the S.noctilio mucus which were used in their investigations. This does not imply that mucus from the mucus reservoir will necessarily contain secretions from other glands besides the mucus gland, but in the process of removing the mucus reservoir from the female wasp, contamination

with other body secretions may have occured.

S.noctilio mucus was shown to consist of 15 protein bands, of which three were cathodic on starch gel electrophoresis (Gaut, 1970). Phenoloxidase, catalase, peroxidase, β-esterase, alkaline phosphatase and ATPase were not detected, but α-esterase and acid phosphatase were present. The most active of these enzymes, acid phosphatase, consisted of six isoenzymes. The enzymes were labile to heat.

The biological activity of S.noctilio mucus was shown to be stable to heat (Coutts, 1969), and therefore was not dependent on the presence of these enzymes. A mucus solution which is very viscous when freshly prepared becomes watery after autoclaving; in the first part of this thesis it was shown that autoclaved mucus caused an earlier senescence of P.radiata needles than did raw mucus, and this was partly due to the faster rate of uptake of the autoclaved mucus solution. For the sake of easier handling, and due to the fact that its biological activity is stable to heat, most of the following investigations are carried out on an autoclaved mucus solution.

Methods

a. Preparation of mucus solution

Mucus glands dissected from adult female wasps upon emergence from P.radiata logs, were stored in the freezer until required. A weighed amount of mucus (thawed) was dissolved in deionised water by continuous stirring at 2°C, to yield a viscous preparation from which tissue debris was removed by filtration through glass wool. When required, solutions were autoclaved at 1 atmosphere pressure (i.e., 15 p.s.i.) for 15 min.

b. Absorption spectrum of mucus solution

A preparation of raw or autoclaved mucus solution (0.5% in deionised water) was placed in a silica cuvette and its absorption spectrum scanned over the range of 190nm to 600nm, with respect to deionised water. An acid hydrolysed mucus solution (hydrolysed in 0.4N HCl for 24 hours at 100°C, and clarified by filtration on a gooch filter) was similarly used for measurement of absorption spectrum.

c. Detection of specific functional groupings

A range of simple chemical tests for the detection of specific functional groupings in S.noctilio mucus, were carried out according to procedures set out in Hawk, et al (1947) and Dawson, et al (1959). Details of these tests are given in the appendix.

d. Reaction to histochemical stains

Solutions of autoclaved mucus (1%, 5% or 10%, in deionised water) were combined with basic dyes in the range of pH 1 (i.e., 0.1N HCl), pH 3 (i.e., 3% HAc), pH 4.5, 5.0, or 5.6 (i.e., sodium acetate buffer, 0.1M), and pH 7 (i.e., deionised water). The basic dyes used were:

i) Toluidine blue (Flatters and Garnet, Ltd.). The absorption spectrum was determined for the mucus - dye complex in different pH solutions. For comparison, chondroitin sulphate "C" from shark cartilage (Calbiochem) was used.

ii) Alcian blue 8GX (Gurr). Anionic groups which combined with alcian blue were differentiated with increasing concentrations of $MgCl_2$ (0 - 1.0M), according to Scott and Dorling (1965). The reagent used for this purpose consisted of 0.05% alcian blue in pH 5.6 sodium acetate buffer, 0.02M, in varying concentrations of $MgCl_2$. A drop of 10% autoclaved mucus applied as a dried film on a glass slide was incubated in the reagent for 1 hr at room temperature, and visualized after

a quick rinse in deionised water.

iii) Acridine orange (Gurr). Differentiation of anionic groups with increasing concentrations of NaCl was carried out according to Saunders (1964).

Other histochemical stains used were amido black for protein and periodic acid - Schiff reagent for carbohydrate (Chayen, et al, 1969).

e. Viscometry

Viscosity was measured in a Cannon-Fenske type of glass viscometer of 4ml bulb capacity and 0.6mm bore capillary. The time taken for 9ml of solution to pass between two marked positions on the viscometer, at 25°C, was taken as the "flow time" and used as an indication of the relative viscosity of that solution. Viscosity measurements were made of an aging and autoclaved mucus solution (0.5%); the effect of NaCl, KCl, $CaCl_2$ and $CuSO_4$ on the viscosity of a freshly prepared mucus solution was also investigated.

f. Aging of mucus solution

A freshly prepared solution of mucus (2%) was delivered into plastic vials with caps. Half of the vials was exposed to ultraviolet light at 2560A for 1 hr in an enclosed box; during this period of UV treatment, the temperature of the enclosed box was observed to change from 21°C (room temperature) to 26°C. The controls were left standing at room temperature for the same length of time. The vials were then transferred to an incubator set at 37°C. At specified time intervals, the UV treated and control vials were removed from the incubator and used for analyses. Samples which were not assayed immediately were stored in the freezer. Changes to the level of protein, aminoacid, carbohydrate, reducing sugar and enzymes were measured in these samples.

g. Effect of dialysis

Cellulose tubings (Visking Co.) of size 8/32 and flat width 25/64 were soaked in hot water for three minutes, gently eased open, and immersed in deionised water. One end of each tubing was tied with a double knot, and the free end was attached to a glass tube fitted to the rubber stopper of a 1000ml - capacity Buchner flask. To the Buchner flask was added 700ml of deionised water. The rubber stopper with attached dialysis bag was set in place and suction applied to fully inflate the dialysis bag; excessive suction pressure causes the dialysis bag to over-inflate and burst and is to be avoided. The vacuum in the flask was maintained by tightening the screw clip on the arm of the flask. Each sample consisting of 3ml of 5% mucus solution (fresh or aged at 37°C) was delivered into the dialysis bag through the glass tube, and the whole apparatus was stored at 4°C for 50hr. During this period, three changes of deionised water from the flask were made. At the conclusion of dialysis the retentate solution was adjusted to 3ml and used for chemical and bio-assays.

h. Detection and characterization of enzymes of S.noctilio mucus

i) Amylase

The pH regime for optimal amylase activity was determined with a 1% starch solution in acetate buffer (0.1M) or phosphate buffer (0.1M). Maltose released from the digestion of starch at 37°C, for 5min, was quantitated by the method of Bernfield (1955).

ii) Esterase

a) effect of substrate and pH on the esterase activity

The substrate solutions used were:

α-NA, 0.02% in acetate buffer, 0.1M, pH5; or phosphate buffer,

0.1M, pH 6.4.

∝-NP, 0.02% in acetate buffer, 0.1M, pH 5; or phosphate buffer, 0.1M, pH 6.4.

p-NPP, 0.02% in acetate buffer, 0.1M, pH 5; or phosphate buffer, 0.1M, pH 6.4.

A 2% fresh mucus solution was first electrophoresed on 8% acrylamide gel, and the electrophoretograms incubated in the substrate solutions for ½hr at room temperature. Following the addition of an aqueous solution of Fast Blue BB to the incubation mixture, the electrophoretograms were further incubated for 1hr. The relative staining intensities of the electrophoretograms in different substrate solutions were assessed visually.

b) differentiation of A- and B- esterases

The following reagents were used in the reaction:

Inhibitors: aqueous solutions of eserine or PCMB at 2 X 10^{-4}M (i.e., carbamate and mercurial compounds, respectively), and Malathion at 0.01% (i.e., organophosphate compound).

Substrate: 0.02% ∝-NA in phosphate buffer, 0.1M, pH 6.4.

Diazo - coupling reagent: 0.04% aqueous solution of Fast Blue BB.

To 0.2ml of 2% aqueous solution of mucus was added 0.2ml of inhibitor or 0.2ml of deionised water. After 30min at 25°C, 2ml of substrate solution and 0.5ml of diazo - coupling reagent were added, and the mixture incubated at 25°C for 1hr. The reaction was terminated by immersing the reaction tube into a boiling water bath for 5min. After cooling, the optical density of the solution was read at 440nm.

c) detection of phosphatase activity

Substrate for acid phosphatase: 0.02% ∝-NP in acetate buffer,

0.1M, pH 5.5, containing 5mM of $MgCl_2$.

Substrate for alkaline phosphatase: 0.02% α-NP in carbonate buffer, 0.1M, pH 10, containing 5mM of $MgCl_2$.

A 0.2ml of 2% mucus solution was incubated with 2ml of substrate solution for 1hr at 25°C, and the reaction was halted by immersing the reaction tube in a boiling water bath for 5min. After cooling, 0.5ml of deionised water was added and the absorption spectrum of the purple coloured mixture was determined.

iii) Phenoloxidase

a) substrate specificity

The following substrates were made up in 0.1M acetate buffer, pH 4.5: o-dianisidine (1.25×10^{-3}M), guaiacol (20×10^{-3}M), catechol (20×10^{-3}M), p-phenylenediamine (20×10^{-3}M), hydroquinone (20×10^{-3}M), p-cresol (20×10^{-3}M), and tyrosine (20×10^{-3}M).

A 0.02ml of 0.2% mucus solution was added to a silica cuvette containing 1ml of substrate solution and 1ml of deionised water. The mixture was quickly stirred, and the reaction at 440nm was recorded on a chart recorder which was attached to the spectrophotometer. The mucus, reagents and spectrophotometer were maintained at 25°C.

b) inhibitors of phenoloxidase activity

Using catechol as substrate, the phenoloxidase activity of Sirex mucus was measured in the presence of an inhibitor, in the usual manner. Aqueous solutions of the following inhibitors (at 5×10^{-3}M) were prepared: sodium diethyldithiocarbamate, semicarbazide-HCl, hydroxylamine, sodium azide, and H_2O_2.

iv) Proteolytic enzyme

Enzymic activity was monitored with 1% solidified gelatin or with

Azocoll, in phosphate buffer, 0.01M, pH 7.4, at 37°C.

i. Procedures of isolation and purification

i) Precipitation with 80% acetone at 2°C

Raw mucus was precipitated with four volumes of 80% aq acetone at 2°C, and the white precipitate collected by filtration. Both precipitate and filtrate were evaporated to dryness over a stream of air and redissolved in deionised water. The absorption spectra of these two solutions were determined over the range of 190nm to 800nm, and subsequently used for gel filtration and bioassays.

ii) Isolation with 40% aq phenol at 60°C

Raw mucus (0.5g) was dissolved in 10ml of 0.2M NaCl and stirred with 100ml of 40% aq phenol at 60°C for 1hr. The mixture was transferred to a 4°C room overnight, and the three layers were separated by centrifugation. After drying by evaporation in a stream of air, each of the three layers was dissolved in deionised water and used for the following analyses:

a) detection of protein with the Millon and Biuret reagents; and carbohydrate with the anthrone and Benedict's reagents.

b) sugar analysis by means of thin layer chromatography.

c) the mucus precipitate present in the interphase layer was dissolved in deionised water and reprecipitated with two volumes of absolute ethanol at 2°C. An aqueous solution of this precipitate was then autoclaved and fractionated on Biogel P6. Both eluent fractions and column residues were bioassayed.

iii) Precipitation with quaternary ammonium salts

Cetylpyridinium chloride was used for the precipitation of proteolytic digests of autoclaved mucus, according to the procedure of

Schiller, et al (1961). Details of this procedure are given in the appendix. The Sirex mucus was isolated from its insoluble complex with cetylpyridinium chloride, and then subjected to ion-exchange chromatography on AG1 X2 (Cl^- form). The eluent fractions were monitored for protein and hexose, some of the fractions were electrophoresed, and they were finally analysed for sugars by TLC and for sulphate.

iv) Filtration with acrylamide gels

Acrylamide gels obtained in the form of Biogels (P series) from Bio-Rad laboratories, California, were chosen for their different nominal molecular weight exclusion limits (in parenthesis): P2 (1,600), P6 (4,600), P10 (10,000), P30 (30,000), P60 (60,000), and P100 (100,000). The gels were swollen in deionised water overnight, and then packed into a precision bore borosilicate column of 48ml capacity (Pharmacia, Sweden). The void volume of the column was determined with dextran blue which has a molecular weight of 1,000,000.

A 4% mucus solution (raw, autoclaved or aged at 2^oC) of 2ml or 4ml, was admitted from the base of the column and eluted with deionised water by an upward flow procedure by means of a peristaltic pump, at 2^oC or at room temperature. 54 drops (i.e., 1.5ml) were delivered into each tube by the distributor of an automatic LKB fraction collector (Sweden). Each of the fractions was monitored for carbohydrate (with the anthrone reagent), protein (with the Folin-Ciocalteau reagent), amylase and phenoloxidase. Some of the fractions were also examined by electrophoresis. Fractions combined according to the positions of their protein and carbohydrate peaks were bioassayed.

v) Ion - exchange chromatography

Anion - exchange resins, AG1 X2, 200 - 400 mesh (Cl^- form) and

DEAE - Sephadex, 200 - 400 mesh (Cl^- form) were prepared (refer to the appendix for detailed procedures) and packed into precision bore borosilicate columns of 48ml capacity. Autoclaved mucus solutions were eluted from the columns by an upward flow procedure with tris-HCl buffer, 0.1M, pH 7.4, containing NaCl either in the form of a continuously increasing straight gradient or in a stepwise - increasing concentration. 1.5ml or 2.0ml fractions were collected by the automatic LKB fraction collector, and the fractions were monitored for protein (with the Folin-Ciocalteau reagent), hexose (with phenol - sulphuric acid), and hexuronic acid (with the modified carbazole reagent). Some of the fractions were examined by electrophoresis; the rest of the eluent fractions were combined according to the positions of protein and carbohydrate. Aliquots of these solutions were either hydrolysed in HCl and subsequently used for TLC analyses of sugars and aminoacids, or bioassayed.

j. Electrophoresis

i) preparative scale electrophoresis

The technique of slab electrophoresis in a discontinuous buffer system was employed. 6% acrylamide gel (i.e., "Cyanogum" 41) was polymerised with DEAE - cyanide and ammonium persulphate, in tris - citrate buffer, pH 8.6, in a rectangular template of dimensions 18.2cm (length), 5cm (breadth), and 1.8cm (thickness) (Shandon). A trough of surface area 3cm X 0.7cm was cut into one end of the gel, and into this trough was introduced a mixture of acrylamide gel macerates containing 2ml of 4% aged mucus solution (i.e., stored at $2^{\circ}C$ for 12 weeks). Bromophenol blue was introduced into slits, made on either side of the mucus trough. The slab of gel was then positioned into the electrophoresis tank con-

taining borate buffer, pH 8.6, with strips of borate buffer - saturated muslin forming the bridge between the gel and the tank buffer. Electrophoresis was conducted at 5°C in a constant current of 14mA for 18 hours. During this period of time, the bromophenol blue dye was located 12cm from the origin. A blank gel was subjected to electrophoresis in the same manner.

A slice of gel, about 2mm thick, was cut from the thick slab and stained with amido black for protein. The remainder of the slab of gel was cut into 3cm strips and macerated in 3ml aliquots of deionised water. The gel extracts were collected by suction filtration, and filtrates assayed for protein by the method of Lowry, et al (1951).

ii) semi - micro scale electrophoresis

Electrophoresis on 8% acrylamide gel in a discontinuous buffer system was carried out according to the procedure of Mills and Crowden (1968), and given in detail in the appendix. Each electrophoretogram was sliced into four thin slices with a Shandon gel slicer, and stained with one of the following: amido black for protein, periodic acid - Schiff reagent for carbohydrate (Keyser, 1964), I_2/KI for amylase, α-NA and Fast Blue BB for esterase, o-dianisidine for phenoloxidase, and toluidine blue (Chayen, et al, 1969), alcian blue (Steedman, 1950), and acridine orange (Saunders, 1964) for acid mucopolysaccharides. The study of enzymes, namely of amylase, required the incorporation of 0.6% starch into the 8% acrylamide gel during its preparation.

k. Thin layer chromatography

A standard sample of mucus was used for hydrolysis in each case. For this purpose, 0.16g of autoclaved mucus was passed through a Biogel P6 column and eluted with deionised water. The eluent fractions

were evaporated to dryness over a hot plate in a constant stream of air, and used for acid hydrolysis under the following conditions:

i) Reducing sugars and hexosamines

The mucus residue was hydrolysed in 5ml of 8N HCl at 100°C for 2hr, in a sealed ampoule. The acid was evaporated off, and the residual hydrolysate was passed through a 25ml column of Dowex 50W - 4X, 200 - 400 mesh (H^+ form). Reducing sugars were eluted from the column with 50ml of deionised water, and was followed by the elution of hexosamines with 50ml of 1N HCl. The HCl eluent fractions were assay-for hexosamines according to the method of Boas (1953).

Both H_2O and HCl eluent fractions were then evaporated to dryness and redissolved in 10% isopropanol for TLC on cellulose powder (Whatman CC 41), 25μ thick.

Conditions for chromatography of reducing sugars:

Solvents: n-butanol - ethanol - water (4 : 1 : 1, v/v); abbreviated as BEW.

or ethylacetate - acetic acid - water (3 : 1 : 1, v/v); abbreviated as EAW.

or n-butanol - pyridine - water (6 : 4 : 3, v/v); abbreviated as BPW.

Spray reagents: aniline hydrogen phthalate (Zweig and Whitaker, 1967), or aminobiphenyl (Block, et al, 1958).

Conditions for chromatography of hexosamines:

Solvent: n-butanol - pyridine - water (6 : 4 : 3, v/v).

Spray reagent: Elson - Morgan reagent (Partridge, 1948).

Chromatograms of hexosamines from the mucus hydrolysates were often streaky in the positions of glucosamine and galactosamine. Further

confirmation was therefore achieved with the ninhydrin degradation of both mucus hydrolysate and authentic hexosamine, according to the procedure of Stoffyn and Jeanloz (1954). The resulting pentoses were separated chromatographically according to procedures laid out for reducing sugars.

ii) Hexuronic acids

The mucus residue was hydrolysed in 5ml of 0.1N HCl at 100°C for 2hr, in a sealed ampoule. The acid was then evaporated off, and the residual hydrolysate was passed through a column of Dowex 50W (H^+), 25ml, and eluted with 50ml of deionised water. The eluent fractions were evaporated to dryness, redissolved in 10% isopropanol, and used for TLC. Conditions for chromatography of hexuronic acids were:

Solvent: n-butanol - pyridine - water (6 : 4 : 3, v/v).

Spray reagent: aminobiphenyl (Block, et al, 1958).

iii) Aminoacids

The mucus residue was hydrolysed in 5ml of 6N HCl at 100°C for 18hr, in a sealed ampoule. After evaporation of the acid, the residue was dissolved in deionised water and passed through two successive ion exchange columns:

1) Dowex 50W - X4, 200 - 400 mesh (H^+ form), 25ml column. The eluent was 1N NH_4OH. The eluent fractions were pooled, evaporated to dryness, redissolved in deionised water, and passed through the next ion exchanger.
2) AG1 - X2, 200 - 400 mesh (Ac^- form), 25ml column. Aminoacids were eluted with 1N HCl. The eluent fractions were evaporated to dryness, redissolved in 10% isopropanol, and used for two dimensional TLC on cellulose powder (Whatman CC 41), 25μ thick.

Conditions for two dimensional chromatography of aminoacids:

Solvent systems: I/II or I/III, according to von Arx and Neher (1963). These solvents are:

I = n-butanol - acetone - diethylamine - water (40 : 40 : 8 : 2, v/v)

II = sec-butanol - methyl ethyl ketone - dicyclohexylamine - water (40 : 40 : 8 : 20, v/v)

III = isopropanol - formic acid - water (80 : 4 : 20, v/v)

Spray reagent: Ninhydrin - collidine - acetic acid (Brenner and Niederwieser, 1967).

1. Assay procedures (details of these procedures are given in the appendix)

Protein: with the Folin - Ciocalteau reagent of Lowry, et al (1951).

Aminoacids: with ninhydrin, being a modified method of Moore and Stein (1954).

Total carbohydrate: with the anthrone reagent (Trevelyan and Harrison, 1952).

Hexose: with phenol - sulphuric acid (Dubois, et al, 1956).

Hexuronic acid: with the modified carbazole reagent of Bitter and Muir (1962).

Hexosamine: with the Elson-Morgan reagent (Boas, 1953).

Sialic acid: with the direct Ehrlich method (Werner and Odin, 1952), and the thiobarbituric acid method (Warren, 1959).

Sulphate: with the spectrophotometric method of Iwasaki, et al (1957), and a modified turbidimetric method of Dodgson and Price (1962). The mucus was first hydrolysed in HCl, under the same conditions as hydrolysis for the

release of aminoacids, and HCl was evaporated from the hydrolysate before it was used for sulphate determination.

Bioassay: excised twigs or individual fascicles from P.radiata trees previously typed for susceptibility to Sirex mucus were used. The plant material was allowed to take up the test solution in the phytotron, and the degree of senescence was determined visually. A scale of senescence from slight (+) to severe, including foliage death (5+), was adopted.

m. Effect of enzymic digestion on the elution profile and biological activity of S.noctilio mucus

i) Raw mucus

4ml of 4% mucus solution was digested with trypsin (pancreas), (0.002 + 0.002)g in 0.2ml of phosphate buffer, 0.1M, pH 7.4, at 37°C for 24hr. The digest was briefly boiled before being fractionated on a Biogel P60 column. It was eluted with deionised water by an upward flow procedure at 2°C. The position at which trypsin was eluted from the column was determined with a sample of denatured trypsin.

ii) Autoclaved mucus

4ml samples of 4% autoclaved mucus were treated with the following enzymes prior to their fractionations on columns of Biogels P6 or P10. The enzymes used were:

a) Trypsin, (0.001 + 0.001)g, in (0.2 + 0.5)ml of phosphate buffer, 0.1M, pH 7.4, at 37°C, for periods of 24hr, 36hr, or 48hr.

b) Papain (African papaya), (0.001 + 0.001)g, in (0.5 + 0.5)ml of acetate buffer, 0.1M, pH 5.5, containing 0.005M cysteine, at

37°C, for periods of 24hr or 48hr.

c) Cellulase, (0.003 + 0.002)g, in (0.5 + 0.5)ml of acetate buffer, 0.1M, pH 5, at 37°C, for periods of 24hr, 36hr or 48hr.

The digests to be fractionated at 2°C were first denatured with heat. A sample of papain digest (24hr digestion) was also eluted from Biogel P6 with tris - HCl buffer, 0.1M, pH 7.4; for comparison, an untreated autoclaved mucus solution was similarly eluted from the gel column. Some of the eluent fractions were examined by electrophoresis, and finally bioassayed.

The eluant fractions of a 24hr - cellulase digest, from Biogel P10, were reduced to a total volume of 3ml and refractionated on a column of DEAE - Sephadex, at 2°C. After their dialysis against deionised water, the two major fractions obtained were bioassayed.

n. Effect of enzymic digestion on the histochemical reactions of autoclaved mucus

i) with diastase

0.2ml of a 1% autoclaved mucus solution was digested with 0.2ml of aqueous diastase (0.1%) for 2hr at 37°C. The reaction was terminated by immersing the reaction tube in boiling water. 0.1ml of the digest was then subjected to electrophoresis on 8% acrylamide gel and the electrophoretogram stained with alcian blue, toluidine blue and P.A.S. The rest of the digest solution was allowed to react with toluidine blue and P.A.S., <u>in vitro</u>.

ii) with hyaluronidase (from bovine testes)

0.1ml of a 10% autoclaved mucus solution was digested for 24hr, at 37°C, with 0.001g, 0.0005g, or 0.0002g of hyaluronidase, in 0.1ml of acetate buffer (0.2M, pH 5). The reaction was terminated by immers-

ing the reaction tube in boiling water. As control, a sample of autoclaved mucus in acetate buffer was treated in a similar manner. The products of these treatments were examined by electrophoresis on 8% acrylamide gel, and stained with amido black, P.A.S., alcian blue, toluidine blue, and acridine orange.

o. Studies of mucus secretions from other siricids

Aqueous solutions of mucus from Urocerus gigas and Xeris spectrum were fractionated on columns of Biogels, P6 or P60, and then used for bioassays. Where necessary, the insoluble mucus was first digested with papain before fractionation.

Results

Description of mucus

Mucus obtained from live insects is a clear, gelatinous and sticky substance which turns brown and emits an unpleasant odour when left standing for several days at room temperature. On drying in the oven, fresh mucus turns pale yellow, hard and brittle, and the residue constitutes 68% of the wet weight. A single large wasp may yield as much as 25mg of mucus.

Raw mucus dissolves in deionised water to form a viscous solution of pH 6.5, for a 2% w/v solution, at 20°C. It does not dissolve in acetate buffer at pH 5.0, but is readily soluble in alkali. Mucus is not coagulated by heat. On precipitation with cold acetone or ethanol, the white mucus precipitate redissolves with difficulty in deionised water.

Absorption spectrum

A fresh aqueous mucus solution showed a weak absorbance in the region of 290nm (fig. 30a). This peak of absorbance was not improved

by autoclaving. After acid hydrolysis, the mucus hydrolysate showed a wider band of absorption which extended from 260nm to 290nm (fig. 30b); and is probably due to the release of aromatic rings. There was no absorption in the region of 330nm to 600nm, and material extracted with acetone or ethanol did not show any significant absorption in either ultraviolet or visible light.

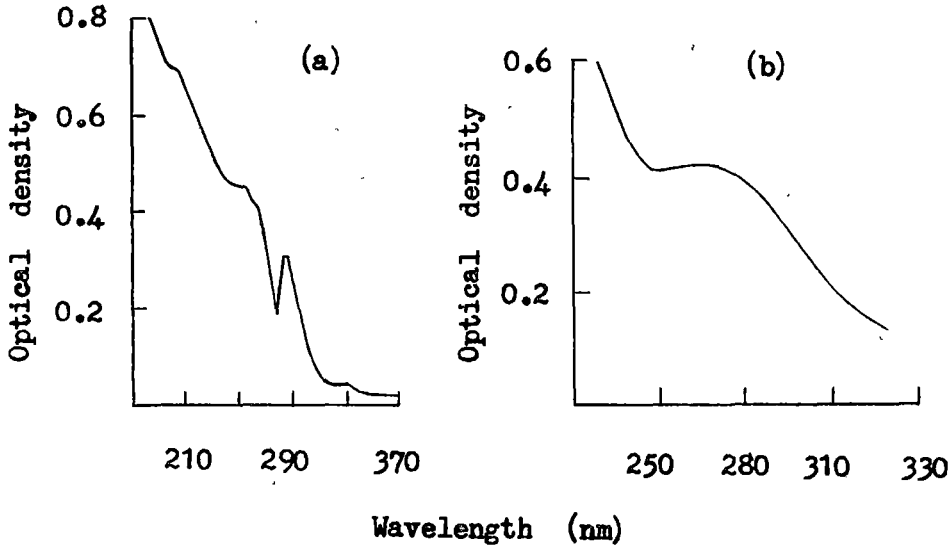


Fig. 30. Absorption spectrum of mucus solutions in ultraviolet light.
a) fresh mucus solution
b) acid hydrolysed mucus solution

Detection of specific functional groupings

Results of simple chemical tests on fresh and aged mucus (table 3) show the presence of both protein and carbohydrate. However, diff-

erences in their reactions to some of the tests (i.e., tests for amino-acids, indoles, aromatic amines and reducing sugars) indicate that these groupings are more readily accessible for chemical reactions in the aged preparation than in the freshly prepared solution. The carbohydrate present in S.noctilio mucus is neither starch nor glycogen.

Table 3: Chemical groupings present in solutions of fresh or aged mucus.

	Reagent	Groups tested for	Mucus solution	
			Fresh	Aged
1.	I_2/KI	starch or glycogen	-	-
2.	Anthrone	carbohydrate	+	+
3.	Benedict's	reducing sugar	+ (very slow)	+
4.	Excess alcohol	precipitation of protein and polysaccharide	+	+
5.	Biuret	protein	+	+
6.	Ninhydrin	amino acids	-	+
7.	Xanthoproteic	aromatic nucleus in protein	-	+
8.	Ehrlich	indoles	-	+
9.	Alkaline Folin-Ciocalteau	phenols and amines	+	+
10.	$CuSO_4.5H_2O$	amines	-	-
11.	Millon's	aromatic amines	-	+
12.	$FeCl_3$	phenols (-SH group)	-	-
13.	Basic lead acetate	-SH groups	-	-
14.	Hopkins - Cole	tryptophan	weak, +	+

Reaction to histochemical stains

1. Periodic acid - Schiff reagent (abbreviated P.A.S.)

A solution of autoclaved mucus reacted with P.A.S. to produce a strong magenta colour, but omission of periodic acid from the procedure resulted in a negative reaction. The protein bands of autoclaved mucus (refer fig.53) were all reactive towards P.A.S.. This reaction was not affected by prior treatment of autoclaved mucus with diastase or hyaluronidase.

The P.A.S. reaction depends on the oxidation (with periodic acid) of two adjacent unsubstituted hydroxyl groups or of one hydroxyl group which is adjacent to a primary or secondary amino group, and subsequent attachment of the Schiff reagent which then colours the compound red or purple - red. Polysaccharides, α-aminoaldehydes and ketones, α-amino-alcohols (e.g., serine), diamines, and ethylene linkages present in unsaturated fatty acids which are oxidised by periodic acid, will produce coloured reactions with P.A.S.. Periodic acid oxidation, if confined to 10min at room temperature, will ensure specificity of the P.A.S. reaction, since prolonged exposure of material to periodic acid at high temperatures will cause the oxidation of aldehydes to carboxylic acids and possibly also affect the oxidation of groups other than the specific 1 : 2 glycol group in a similar manner (Pearse, 1968). Moreover, decomposition of periodic acid to ozone in sunlight will cause erroneous results with P.A.S., because ozone is a strong oxidizing agent.

It is evident that autoclaved mucus consists of glycoproteins, without the presence of starch or glycogen. Hexoses and methylpentoses (e.g., fucose) but not hexosamines or hexuronic acids are responsible for the P.A.S. reaction (Pearse, 1968). Thus, pure hyaluronic acid

will not react with P.A.S., but impure preparations will react even after they have been treated with hyalase, rondase, or streptococcal hyaluronidase (Davies, 1952). Whereas heparin and chondroitin sulphate remain negative, heparin monosulphate reacts positively with P.A.S. (Jorpes, et al, 1948). Pearse (1968) reported that chondroitin sulphate was reactive with P.A.S. only after 60 hr of periodic acid oxidation.

2. Toluidine blue (abbreviated TB)

A dilute aqueous solution of TB has bimodal peaks of absorbance at 590 - 600nm and 620 - 630nm (fig. 31). In the presence of autoclaved mucus solution (1% or 5%), TB remained blue in colour and is therefore an orthochromatic reaction. Dye binding activity by autoclaved mucus caused a lowering of the absorption curve (when compared to that of the dye solution alone) in the pH range of 3 to 7. At pH 1, dye binding activity was abolished so that both TB and AM - TB curves became superimposable. Thus, weak acidic groups present in Sirex mucus appeared to be responsible for the orthochromatic reaction. Carboxyl groups of protein do not participate in reactions with basic dyes at pH regimes below 6 (Chayen, et al, 1969), therefore staining of autoclaved mucus at pH 3 or above may be attributed to unsulphated acidic polysaccharides.

By way of comparison, the effect of authentic chondroitin sulphate "C" (i.e., CSC) on the absorption spectrum of TB was also measured. The addition of CSC to a solution of TB caused a change in colour from blue to pink, with a corresponding shift in the absorption spectrum to 550nm, in the pH range of 3 to 7. This is a metachromatic reaction, and is defined by Pearse (1968) as the staining of a tissue component

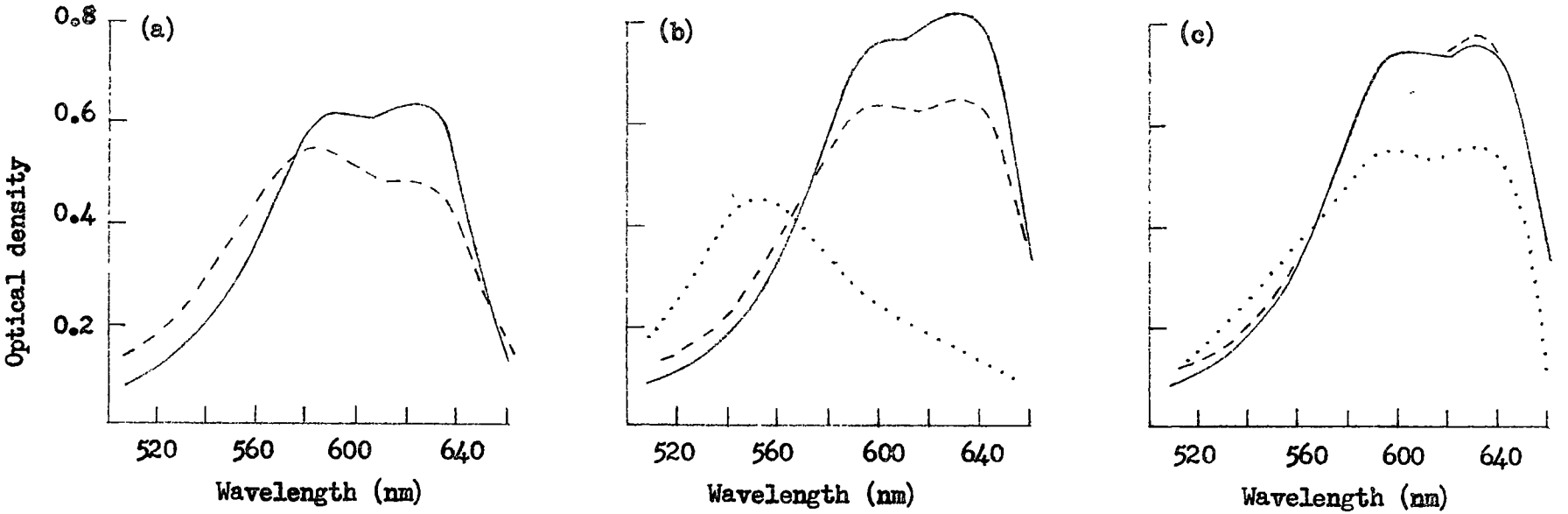


Fig. 31. Absorption spectrum of toluidine blue in the presence of autoclaved mucus or chondroitin sulphate "C".

(a) pH range of 4.5 to 7

(b) pH 3 (i.e., 0.1M acetic acid)

(c) pH 1 (i.e., 0.1M hydrochloric acid)

—— 0.001% toluidine blue solution

– – – solution of 1% AM and 0.001% TB

···· solution of 0.01% chondroitin sulphate "C" and 0.001% toluidine blue

so that the absorption spectrum of the resulting tissue - dye complex differs sufficiently from that of the original dye, and from its ordinary or orthochromatic tissue complexes, to give a marked contrast in colour. At pH 1, the CSC - dye complex was blue in colour and the absorption spectrum was almost similar to that of the dye solution except for a very slight shoulder in the region of 550nm. In spite of the virtual disappearance of the metachromatic peak, dye binding activity by CSC at pH 1 was still evident, and is largely due to the sulphate groups present.

All of the glycoprotein bands of autoclaved mucus stained purple with TB over the pH range of 3 to 7. As purple is formed by the interaction of two primary colours red and blue, the presence of purple mucus bands is not due to the occurence of partial metachromasia, but is the result of mucus molecules having been concentrated into narrow regions by electrophoresis. In solution, a concentrated autoclaved mucus solution (e.g., 5% or 10%) did not react metachromatically with TB. Thus, the interpretation of staining reactions of acid mucopolysaccharides with basic dyes must be made with reference to the relative concentrations of dye and sample, presence or absence of NaCl, and temperature of the solutions used in the reactions (Sylvén and Malmgren, 1952; Schoenberg and Moore, 1964).

Failure to degrade autoclaved mucus with bovine testicular hyaluronidase indicates that hyaluronic acid or chondroitin sulphate are absent. This is contradictory to the typically non - sulphated acid mucopolysaccharide type of staining reaction in TB, given by autoclaved mucus (refer to literature survey on histochemical reactions). Hyaluronidase from mammalian or bacterial sources is not specific for

hyaluronic acid as it also attacks chondroitin sulphates A and C, whereas leech hyaluronidase is by far the most specific enzyme known to degrade hyaluronic acid without having any effect on chondroitin sulphates A and C (Hunt, 1970). For these experiments, bovine testicular hyaluronidase (Calbiochem) was used as leech hyaluronidase was unavailable in spite of attempts at procuring it through the chemical agencies in Tasmania.

The effect of hyaluronidase on the stainability of mucus in TB was also investigated by Boros (1968): two blobs of mucus, one treated with hyaluronidase (source unspecified) the other immersed in 0.85% saline, stained blue with TB. Half an hour later, the colour of the hyaluronidase - treated mucus had disappeared but the colour of the control mucus (in saline) remained unchanged. These results indicated the possible presence of hyaluronic acid, but were nevertheless inconclusive (Boros, 1968). In my own studies of the effect of inorganic salts on raw mucus, I found that low concentrations of inorganic salts had caused the mucus macromolecule to dissociate and lose its viscosity (refer to pages 209 and 210). That Boros' blob of control mucus had remained blue in TB, was possibly due to accessibility of the acid groups from mucus for reaction with the basic dye.

3. Alcian blue (abbreviated AB)

Autoclaved mucus stained blue - green with alcian blue in regions of the electrophoretogram which corresponded with glycoprotein bands, over the range of pH 3 to 7, although staining at pH 3 was very faint. The formation of salt linkages between polysaccharide and protein at this pH (i.e., in 3% acetic acid) is the possible cause of loss of staining (Scott and Dorling, 1965). A positive reaction with alcian blue

confirms the presence of acid mucopolysaccharides, and also confirms the earlier findings of Boros'. Results given in table 4 suggests that carboxyl groups present in autoclaved mucus are responsible for the alcian blue reaction. Table 5, taken from Scott and Dorling (1965) shows the effect of $MgCl_2$ on the reactions of carboxyl, phosphate and sulphate groups with alcian blue.

Table 4: The reaction of autoclaved mucus with AB, in varying concentrations of $MgCl_2$, at pH 5.6.

Autoclaved	Molarity of $MgCl_2$ in alcian blue solution						
mucus (10% solution)	0	0.01	0.02	0.03	0.05	0.10	0.2 - 1.0
Thin film	2+	2+	2+	2+	±	-	-
Electrophoretogram	3+	3+	2+	2+	+	+	-

Table 5: Staining of spots containing polyanions on filter paper in AB - pH 5.8 - $MgCl_2$. (From Scott and Dorling, 1965.)

Type of	Polyanion	Molarity of $MgCl_2$									
anionic group		0	0.025	0.05	0.1	0.2	0.3	0.45	0.65	0.8	1.0
$-COO^-$	Hyaluronate	2+	2+	2+	∓	-	-	-	-	-	-
	Alginate	∓	+	2+	3+	-	-	-	-	-	-
$=PO_4^-$	RNA	∓	+	2+	2+	∓	-	-	-	-	-
	Polyphosphate	∓	+	+	3+	∓	-	-	-	-	-
	DNA	∓	2+	2+	3+	∓	-	-	-	-	-
$-COO^-$ and $-OSO_3^-$	Heparin	∓	2+	2+	3+	3+	3+	3+	3+	±	-
$-OSO_3^-$	Keratan sulphate	2+	2+	2+	3+	3+	3+	3+	3+	3+	+

Alcian blue combines with, and precipitates polyanions in the same way as cationic detergents or quaternary ammonium salts (Scott, 1960; Scott, et al, 1964; Scott and Dorling, 1965). The polyanionic complexes which are formed, are soluble in specific concentrations of salt. Using the method of Scott (1960), Gaut (1970) combined 1ml of 5% S.noctilio mucus with a quaternary ammonium salt (type unspecified) but failed to detect any precipitation in the presence of 0.04M to 8M $MgCl_2$ solution. He concluded that acid polysaccharides were absent. On the contrary, Boros (1968) and myself have shown that acidic polysaccharides are present in S.noctilio mucus, on the basis of histochemical reactions. Moreover, carboxyl groups in the mucus appeared to be responsible for the alcian blue reaction (tables 4 and 5).

On treating a papain - digested autoclaved mucus solution with excess CPC dissolved in 0.04M NaCl solution, some precipitation was observed but the bulk of the mucus apparently remained in solution. Thus, extract number 1 with 0.04M NaCl (table 6), contained a significant amount of mucus (measured in terms of hexuronic acid level). As further extractions of the precipitate (to which was also added Celite) with 0.04M NaCl and 0.1% CPC yielded more mucus, it is suggested that this mucus was adsorbed onto the Celite and had not formed an insoluble precipitate with CPC. The failure of the bulk of the mucus to form a complex with CPC suggests that a significant proportion of mucus is essentially neutral in its properties.

In the presence of 0.4M NaCl and 0.1% CPC, a small amount of mucus was extracted from the precipitate, which suggests that hyaluronic acid may be present (Schiller, et al, 1961). As there was no mucus present in the 1.2M NaCl and 2.1M NaCl extracts, it appears that neither chondroitin sulphate nor heparin was present.

Table 6: Extraction of mucus with NaCl solutions, from a mixture consisting of CPC, mucus, and the insoluble mucus-CPC complex.

Extract number	0.04 M NaCl extracts		0.4 M NaCl extracts	
	(ml)	$O.D._{530}$ (Hexuronic acid)	(ml)	$O.D._{530}$ (Hexuronic acid)
1	10	1.47	6	0.04
2	6	0.50	6	0.0
3	6	0.15	6	0.01
4	6	0.07	6	0.01
5	6	0.04	6	0.0
6	6	0.0	6	0.0
7	6	0.0	6	0.0

4. Acridine orange (abbreviated AO)

All of the glycoprotein bands of autoclaved mucus stained red with acridine orange, over the pH range of 4 to 7. At pH 3, only the first glycoprotein band remained strongly stained whereas the rest of the bands were very faint. Diastase and hyaluronidase had no effect on the intensities of staining of autoclaved mucus in this dye.

The presence of specific concentrations of NaCl in AO were found to inhibit the dye binding activities of certain acid mucopolysaccharides. An "equivalence point" was attained when both the AO and acid mucopolysaccharide absorption curves became superimposable. The equivalence point for hyaluronic acid occured at 0.04M NaCl, chondroitin sulphate at 0.2M NaCl, and heparin at 0.6M NaCl (Saunders, 1964).

The equivalence point for autoclaved mucus was not clearly defin-

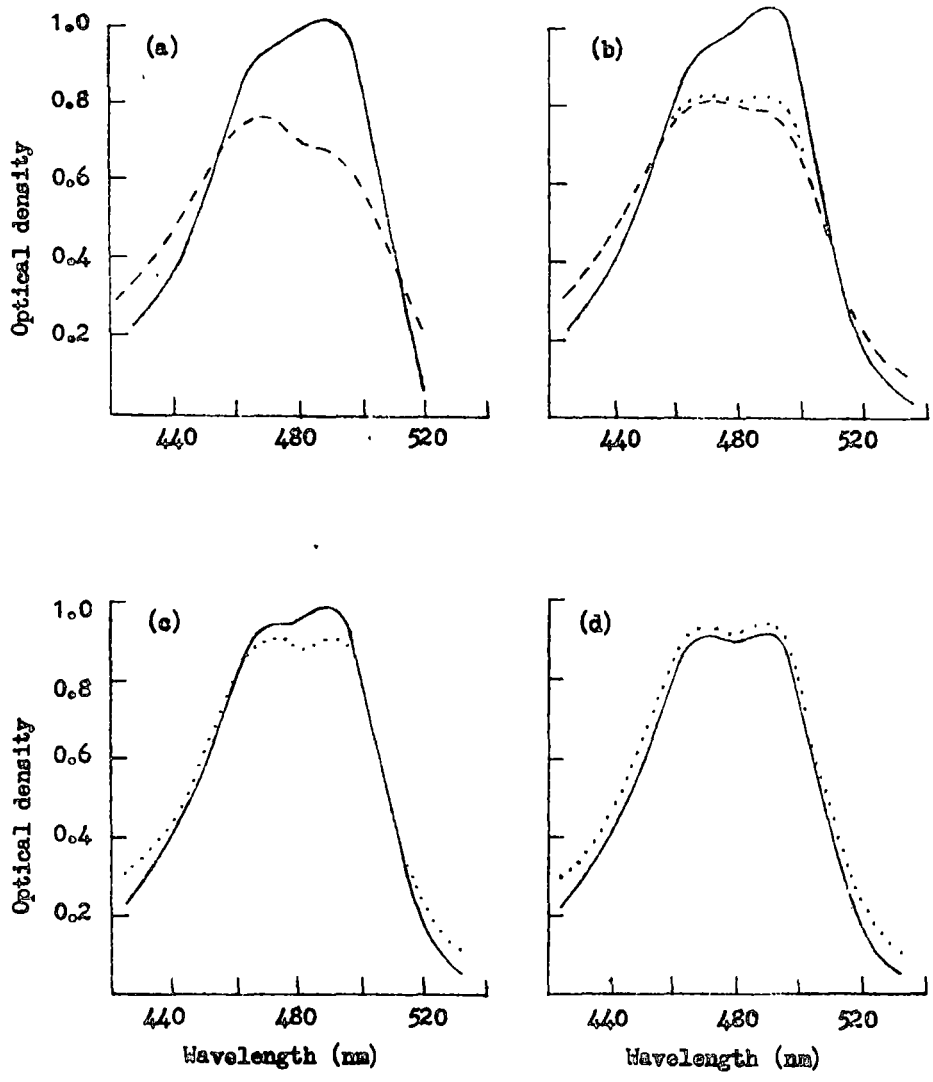


Fig. 32. Absorption spectrum of acridine orange in the presence of autoclaved mucus and varying concentrations of NaCl.

(a) no NaCl. ——AO - - -AO + AM

(b) 0.01M - 0.02M NaCl.

——AO + 0.01M or 0.02M NaCl

- - -AO + AM + 0.01M NaCl

····AO + AM + 0.02M NaCl

(c) 0.1M NaCl. ——AO ····AO + AM

(d) 0.2M NaCl. ——AO ····AO + AM

ed, as the shape of the AO - AM curve was the same at 0.02M to 0.2M NaCl, whereas the shape of the AO curve was variable over this range of NaCl, so that at 0.2M NaCl, both the AO and AO - AM curves were superimposable (fig. 32). However, on comparing the shapes of the curves for AO - AM at 0.0M, 0.01M and 0.02M NaCl, it is apparent that a reduction in dye binding activity had occured as the concentration of NaCl was increased. On this basis, I would suggest that the binding of AO by AM was inhibited in the region of 0.02M NaCl.

On the whole, the reactions of S.noctilio mucus to basic dyes are typical of the reactions of unsulphated acid mucopolysaccharides, and these reactions mostly resemble those of hyaluronic acid. However, several anomalies exist which suggest that S.noctilio mucus is not wholly composed of a hyaluronate type of acid mucopolysaccharide. Thus, it formed an insoluble complex with CPC which was almost completely redissolved in 0.04M NaCl, and its lack of staining with acridine orange in the presence of 0.02M NaCl suggest that neutral polysaccharides may also be present. The presence of these neutral polysaccharides, possibly acting as a shield, render the mucus acid mucopolysaccharide insusceptible to the effects of mammalian hyaluronidase.

Viscometry

A freshly prepared mucus solution is highly viscous, and has a "flow time" of 11min 22sec. In the first 10hr, at 25°C, the flow time was raised to 11min 50sec, but further incubation resulted in a dramatic drop in viscosity so that by 60hr, the viscosity of the aging mucus solution was equivalent to that of autoclaved mucus with a flow time of 3min 30sec. At 200hr, the flow time of the mucus solution was 2min 55sec, which closely approximates that of deionised water (2min

50sec). The initial high viscosity of the freshly prepared mucus solution is indicative of a complex molecular structure. It appears that the chemical bonds of the extended molecule are susceptible to heat, and the severing of these heat - labile bonds, achieved with brief autoclaving would normally require 2½ days of incubation at 37°C, and even longer at lower temperatures. As the viscosity of autoclaved mucus is stable and does not decrease with age, in contrast to that of a raw mucus solution which shows a slow and continuous decline beyond 60hr at 37°C, it is suggested that an enzymatically - induced breakdown process is in operation in the raw mucus solution.

Inorganic salts also caused the mucus macromolecule to disaggregate. Dilute monovalent salts like NaCl or KCl caused the viscosity of a freshly prepared mucus solution to fall, but these effects were not as severe as autoclaving. In the presence of 0.5% NaCl or KCl, the mucus solution had a flow time of 4min 9sec, which is 39sec higher than that of an autoclaved mucus solution. There was no formation of mucus precipitate in the presence of 0.5% NaCl or KCl. These salts, up to 0.5%, had negligible effects on the viscosity of deionised water. The viscosity measurements are given in fig. 33.

Very dilute concentrations of divalent salts like $CaCl_2$ and $CuSO_4$ also exerted dissociative effects on a freshly prepared mucus solution. At 0.04% $CaCl_2$, a slight precipitate of mucus was formed but this precipitate was readily dissolved with vigorous stirring. Beyond 0.4% $CaCl_2$, the mucus precipitated as large gelatinous clumps and the remaining solution became very frothy; this was similarly observed with 0.1% $CuSO_4$. Urea added to a concentration of 8M did not have any similar dissociative effects on S.noctilio mucus, instead, the presence of urea

tended to preserve its viscosity. This is indicative of the absence of hydrogen bonds in the extended structure of the mucus macromolecule. On the contrary, Boros (1968) reported that the shiny surface of a thin smear of mucus turned fluffy after 2hr of contact with 8M urea. This led to the suggestion that hydrogen bonds may have been broken in the process.

Electrophoresis, used as a means of monitoring the salt effect, did not reveal any alterations to the protein banding pattern when compared with raw or autoclaved mucus. The enzymic bands were not affected by the presence of these salts (refer to fig.53a).

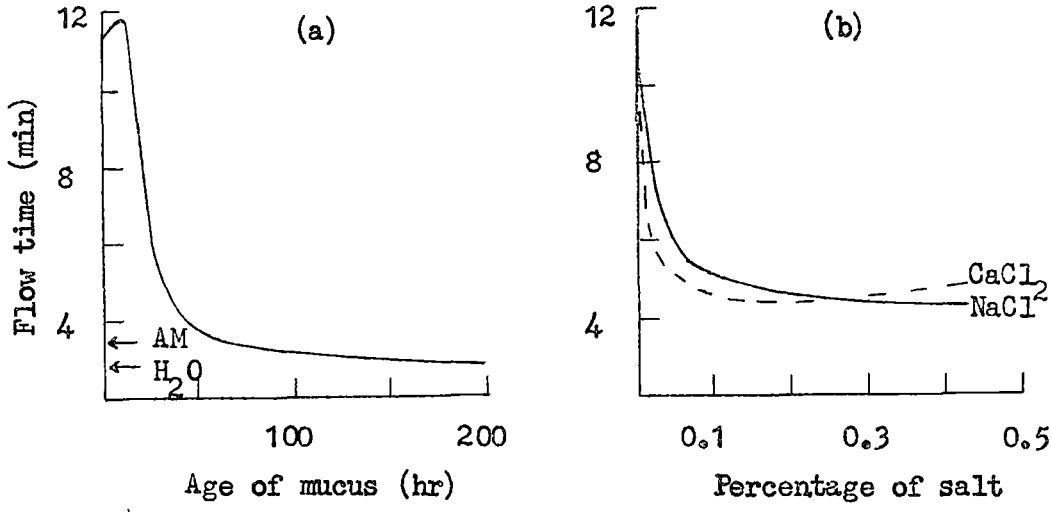


Fig. 33. Viscosity measurements at 25°C.

(a) Relative viscosities (i.e., flow times) of an aging mucus solution, autoclaved mucus solution (arrowed), and deionised water (arrowed).

(b) Effects of increasing salt concentrations on the relative viscosity of fresh mucus solution.

Changes in other properties of S.noctilio mucus, as a result of aging

In the previous section it was shown that aging is accompanied by a dramatic loss in viscosity of the mucus solution. In addition to this loss in viscosity, was the formation of a white precipitate which gave the solution a turbid appearance, about 22hr after its incubation at 37°C.

Changes in the levels of protein, aminoacid, total carbohydrate, reducing sugar, phenoloxidase, amylase, esterase and proteolytic enzyme, are illustrated in fig. 34. Deviations in the amount of change in each of these components were recorded for the UV - irradiated and control mucus solutions. However, evidence of microbial contamination, in the form of new protein or enzymatic bands, were not detected on the electrophoretograms of either the sterilized or unsterilized mucus solutions (fig. 35). This does not imply the lack of microbial contamination, at least in the unsterilized solutions, but that contamination may be present at a very low level.

The rise in protein content for both sterilized and control samples is probably due to the unmasking of reactive sites, brought about by the fragmentation of the macromolecule into smaller units, by an autolytic process involving the mucus' own enzymes. Thus, the formation of three extra fast - migrating protein bands (which probably have lower molecular weights than the native mucus molecule) in the 6hr, 22hr, and subsequent samples (fig. 35a), is correlated with the observed loss in viscosity and increasing turbidity of the mucus solutions. However, the tendency for all of the protein bands, especially the four fast - migrating bands to disappear from the electrophoretograms of samples which were incubated for 190hr or more, may also be the result

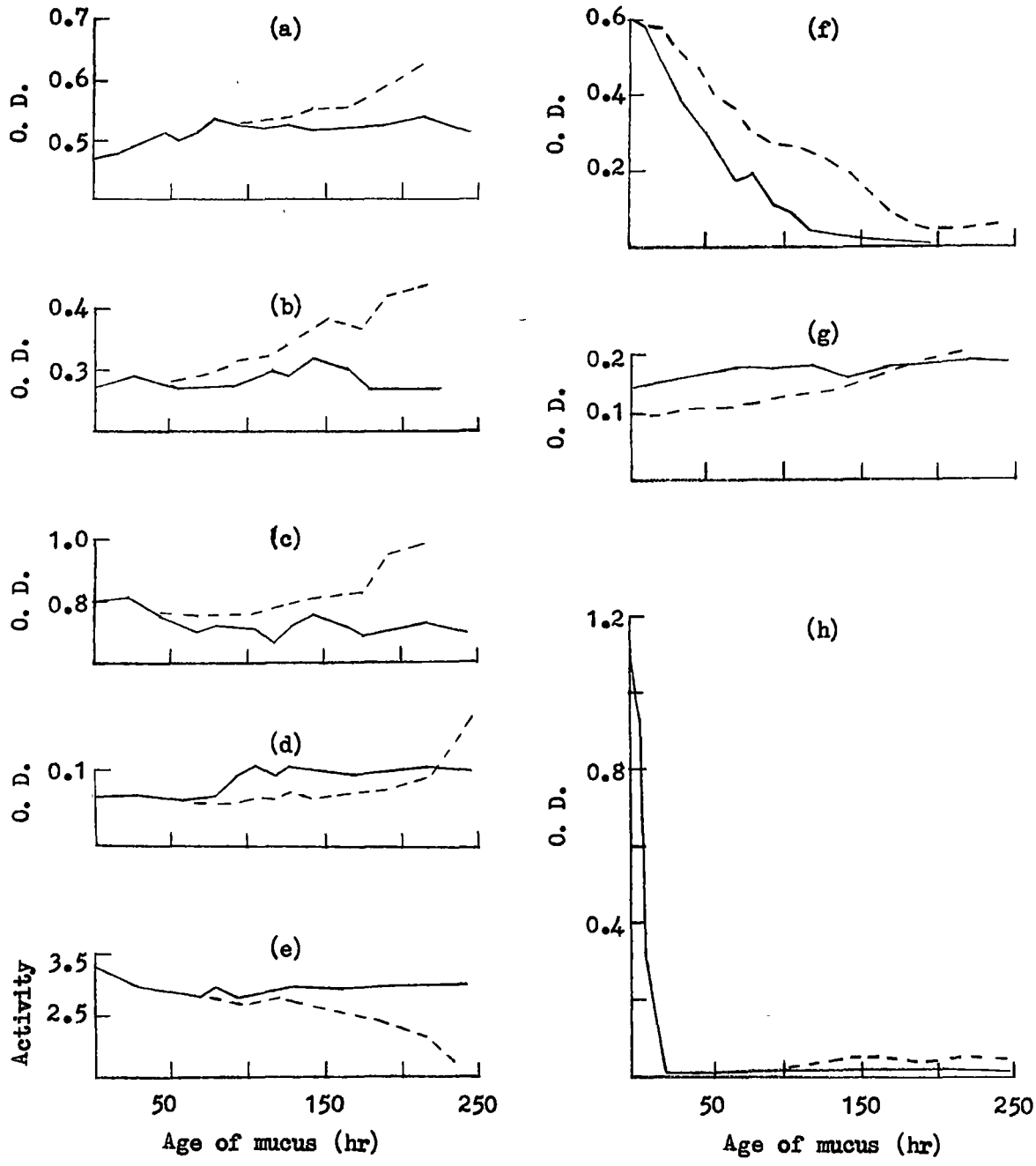


Fig. 34. Effect of aging at 37°C, on some properties of S.noctilio mucus.

(a) protein
(b) aminoacid
(c) total carbohydrate
(d) reducing sugar
(e) phenoloxidase
(f) amylase
(g) esterase
(h) proteolytic enzyme

—— control mucus
--- UV - irradiated mucus

Fig. 35. Electrophoretograms of aged mucus solutions.

a. protein

b. amylase

c. esterase

d. phenoloxidase

Arrangement of samples from left to right, with numbers indicating the age of mucus in hours, at 37°C:

0 6 22 33 55 79 105 127 151 190 245

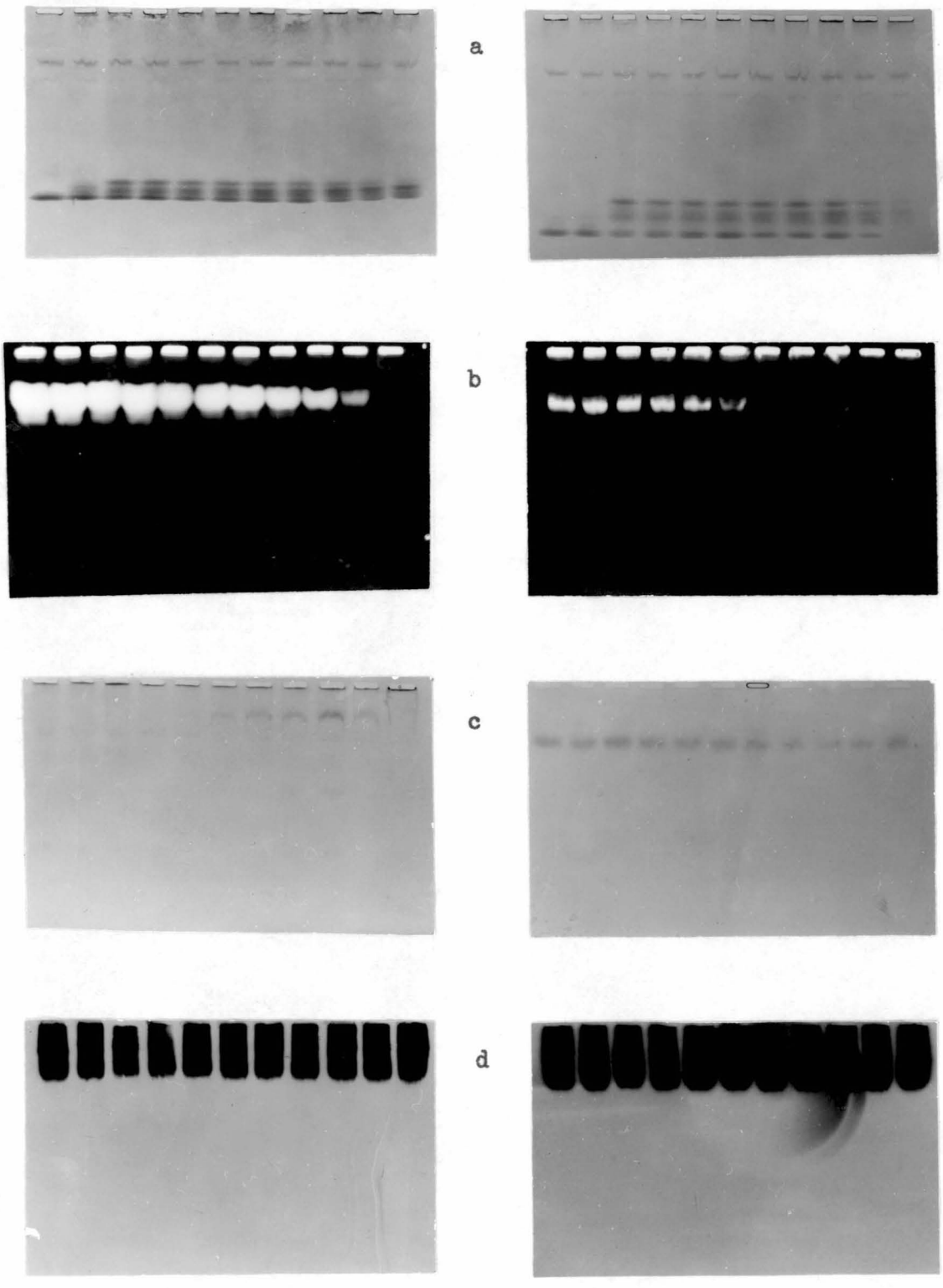


Fig. 35. Electrophoretograms of aged mucus solutions.

of the activity of the mucus' own enzymes. This partly accounts for the earlier observation on the continued loss of viscosity of the mucus solution to one which approximated that of deionised water (fig. 33a). The regions of enzymatic activity on the electrophoretograms remained unaltered, in spite of aging and the reduced activities of all the enzymes except for esterase which showed a quantitative increase in acitivity with aging.

Characteristics of mucus enzymes

Amylase: optimal activity occured in the pH range of 5 - 5.4 (fig. 36), which indicates that the mucus amylase is more like a β- than an α- type of amylase.

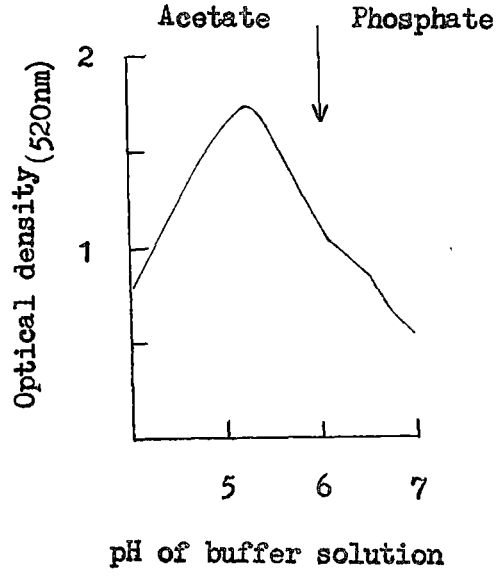


Fig. 36. Amylase activity of S.noctilio mucus in different pH media. Arrow indicates the change over from acetate to phosphate buffer, at pH 6. Activity is expressed in terms of $O.D._{520nm}$.

Esterase: these belong to a class of hydrolytic enzymes which exhibit extensive heterogeneity and have wide substrate specificity. Gen-

erally, esterases can be separated into the A- type esterases which act preferentially on acetate esters, they are resistant to diethyl p-nitrophenylphosphate, and they are not inhibited by organophosphates; and the B- type esterases which act preferentially on butyric esters, and they are inhibited by organophosphates. In mammals, arylesterases preferentially hydrolyse aromatic esters and their reactions are inhibited by PCMB but not by organophosphates; carboxylesterases preferentially hydrolyse aliphatic esters, they are inhibited by organophosphates and not by carbamates like eserine; cholinester hydrolases preferentially act on choline esters rather than on aliphatic or aromatic esters, and they are inhibited by carbamates and organophosphates; acetyl esterases hydrolyse aromatic esters, and their reactions are not affected by organophosphates, eserine or PCMB (Holmes and Masters, 1967). Histochemical detection of esterases generally involves the use of α-naphthylacetate, a substrate for both A- and B- type of esterases, and subsequent precipitation of the product by coupling it with a diazo dye. The esterase activity of S.noctilio mucus was greatest when α-NA at pH 6.4 or α-NP at pH 5 were used (table 7).

Table 7: Intensity of staining of esterase electrophoretograms of S.noctilio mucus, in different combinations of substrate and pH of the buffer medium.

Substrate	pH of buffer solution	Intensity of staining
α-NA	5	trace
α-NA	6.4	2+
α-NP	5	2+
α-NP	6.4	+
p-NPP	5 or 6.4	nil

Although inhibition was not complete, eserine (a carbamate) and PCMB (a mercurial compound) depressed the esterase activity of mucus by 31% and 34%, respectively. Malathion (organophosphate compound) depressed the esterase activity by 12% (table 8). Thus, the crude mucus appears to contain more of the A- type than of the B- type of esterase.

Table 8: The effect of inhibitors on the esterase activity of S.noctilio mucus.

Inhibitors	Esterase activity (O.D. at 440nm)	∴ % of inhibition
Eserine (2×10^{-4}M)	0.07	31
PCMB (2×10^{-4}M)	0.066	34
Malathion (0.01%)	0.09	12
nil	0.10	-

A phosphatase is regarded as a specific type of esterase, as both esterases and phosphatases cleave rather similar ester bonds (Chayen, et al, 1969). Acid phosphatases are actively involved in tissue degeneration, and a high acid phosphatase activity corresponded to the appearance of a maximum number of autophagic vacoules (lysosomes) in the midgut epithelium of cockroaches (Couch and Mills, 1968). In S.noctilio mucus, the acid phosphatase activity was about six times the activity of esterase (table 9). The product of the reaction between mucus acid phosphatase and α-NP was a deep purple colour with a maximum absorption at 530nm (fig. 37); as a result, no diazo dye was

used in the reaction medium. Alkaline phosphatase activity was not detected in S.noctilio mucus.

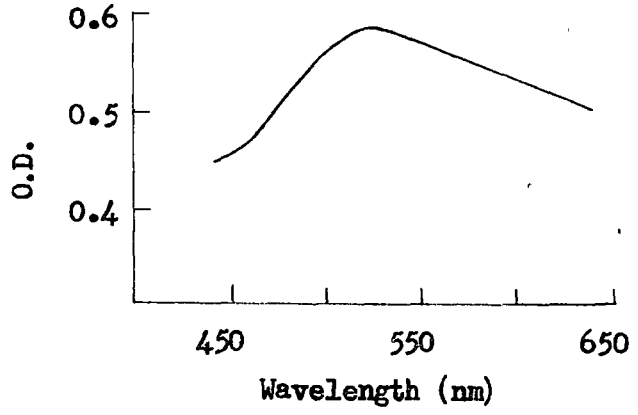


Fig. 37. The absorption spectrum of acid phosphatase from S.noctilio mucus.

Table 9: Activities of acid phosphatase and esterase in S.noctilio mucus. [One unit of activity is defined as the O.D. of the product (at 530nm for acid phosphatase and at 440nm for esterase), derived from the reaction of 0.2ml of 2% mucus solution with 2ml of 0.02% substrate solution for 1hr at 25°C.]

Enzyme	Activity in S.noctilio mucus (Optical density)
Acid phosphatase	0.59
Esterase	0.10

Phenoloxidase: the phenoloxidase activity of S.noctilio mucus was greatest when o-dianisidine was used as substrate (table 10), but there

was no activity in the presence of p-cresol or tyrosine. On comparing the results obtained for S.noctilio mucus with table 11 (taken from Lindeberg and Holm, 1952) for the characterization of fungal tyrosinase and laccase, it is apparent that the phenoloxidase present in the mucus is of a laccase type.

Table 10: Substrate specificity of phenoloxidase from S.noctilio mucus. One unit of activity is expressed as the rate of change of O.D. per minute, derived from the reaction of 0.1ml of mucus solution with 1ml of substrate solution.

Substrate used	Phenoloxidase activity
o-dianisidine	0.25
guaiacol	0.063
catechol	0.05
p-phenylenediamine	0.045
hydroquinone	0.02
p-cresol	0.0
tyrosine	0.0

Table 11: The characteristics of tyrosinase and laccase from extracts of fungal mycelia, taken from Lindeberg and Holm (1952).

	Oxidation of					
Enzyme	catechol	hydro-quinone	p-phenylene-diamine	p-cresol	tyro-sine	Inhibition by CO
Tyrosinase	+	-	-	+	+	+
Laccase	+	+	+	±	-	-

The effect of inhibitors on the phenoloxidase activity of S.noctilio mucus, using catechol as substrate, is given in table 12. The lack of any stimulation in enzymatic activity in the presence of H_2O_2 suggests the absence of peroxidase. Moreover, hydroxylamine, a sensitive peroxidase inhibitor had little effect on the mucus phenoloxidase. The effectiveness of phenoloxidase inhibition in decreasing order is azide, H_2O_2, diethyldithiocarbamate, semicarbazide-HCl, and hydroxylamine.

Table 12: Phenoloxidase activity of S.noctilio mucus in the presence of inhibitors.

Inhibitors used (5×10^{-3}M)	Volume of inhibitor (ml)	Phenoloxidase activity
sodium diethyldithiocarbamate	0.02	0.046
	0.1	0.046
	0.5	0.0
semicarbazide-HCl	0.02	0.056
	0.5	0.038
hydroxylamine	0.02	0.052
	0.5	0.042
sodium azide	0.02	0.0
H_2O_2 (30% aq.)	0.02	0.040
	0.1	0.020
without inhibitor	-	0.052

Proteolytic enzyme: a mucus solution incubated at 37°C for 50hr, has an activity equivalent to 0.015% trypsin for 1g of mucus dissolved in 1ml of deionised water. It has a weak ability to break down gelatin.

Dialysis

The effect of dialysis on the composition of S.noctilio mucus was different for solutions which were fresh or aged. On being dialysed, the aged solutions tended to lose more of their protein and carbohydrate than did fresh mucus (table 13). Moreover, a greater proportion of carbohydrate than protein was lost so that comparisons of the ratio of protein : carbohydrate (i.e., abbreviated P : C) indicated an upward trend with age (table 14). The P : C ratio for fresh mucus was 2 : 1, and for autoclaved mucus was 4 : 1. The high P : C ratio for autoclaved mucus is probably due to the presence of additional reactive protein groups following the breaking of the heat labile bonds of the native macromolecular structure. Both aged mucus solutions had approximate P : C ratios of 3 : 1.

Of the individual types of carbohydrates measured, hexuronic acid also showed a higher percentage of loss through dialysis, with increasing age of the solution. However, a smaller percentage of loss of hexose from the 250hr mucus sample, as compared with a 125hr mucus sample, may be the result of conversion of some carbohydrate to hexose.

The whole mucus solutions were all physiologically active towards P.radiata twigs. The dialysed solutions showed reduced physiological activities; this is related to the loss of physiologically active components through the dialysis bag, as indicated in table 15.

Table 13: The composition of S.noctilio mucus solutions, following dialysis against deionised water at 4°C.

Composition (mg/ml/g fresh mucus)	Fresh mucus			125hr mucus			250hr mucus			AM
	A	B	%	A	B	%	A	B	%	A
Protein	95	87	8	89.5	60	33	82	49.5	40	326
Aminoacid	21.5	-	-	34.5	14.5	58	30	14	53	-
Total carbohydrate	51	19	27	28.5	14.4	50	27.3	12	56	77.5
Hexose	7.3	-	-	5	0.5	93	7.5	1.5	80	-
Hexuronic acid	8	-	-	2.7	2	26	2.9	1.9	35	-

Key: A = undialysed solution; B = dialysed solution

% = percentage of loss of components through dialysis

Table 14: Calculated protein : carbohydrate ratios of S.noctilio mucus.

Mucus solutions	Protein ÷ Carbohydrate	Approximate P : C
Fresh	1.86	2 : 1
Aged for 125hr	3.14	3 : 1
Aged for 250hr	3.0	3 : 1
Autoclaved	4.2	4 : 1

Table 15: Relative physiological activities of test solutions, 14 days after bioassay with P.radiata twigs.

	Degree of twig senescence in mucus solutions			
Test solutions	Fresh	Aged (125hr)	Aged (250hr)	Autoclaved
Whole mucus	5	5	5	5
Dialysed mucus	5	3	3	3
Dialysate	trace	1	1	1

Bioassay of autoclaved mucus with P.radiata twigs

The syndrome of responses to mucus treatment has been described in the first part of this thesis. Two of the most obvious symptoms, loss of chlorophyll and tissue desiccation, were chosen as the criteria upon which visual estimations of rates of senescence were made and coded on a scale of 0 - 5. Needles which did not respond to treatment and appeared to be as healthy as the controls were placed on a scale of 0; needles which turned yellow or brown and dry were placed on a scale of 5 (i.e., needles appeared to be dead).

In the initial screening of 100 plantation trees for susceptibility to S.noctilio mucus, three 1yr old twigs from each tree were each tested against 0.02g of raw mucus. Of these, ten trees were selected for all subsequent bioassays as they were quick to respond to mucus treatment, and therefore showed high susceptibility to mucus. Fig. 38 shows a typical response by the twigs of one of these susceptible trees. The rate of twig senescence appeared to be dependent on the concentration of the mucus present. Whereas a very low concentration of mucus (0.001g) caused mild chlorosis without tissue desiccation, a high concentration of mucus (0.16g) caused rapid browning and drying of the needles.

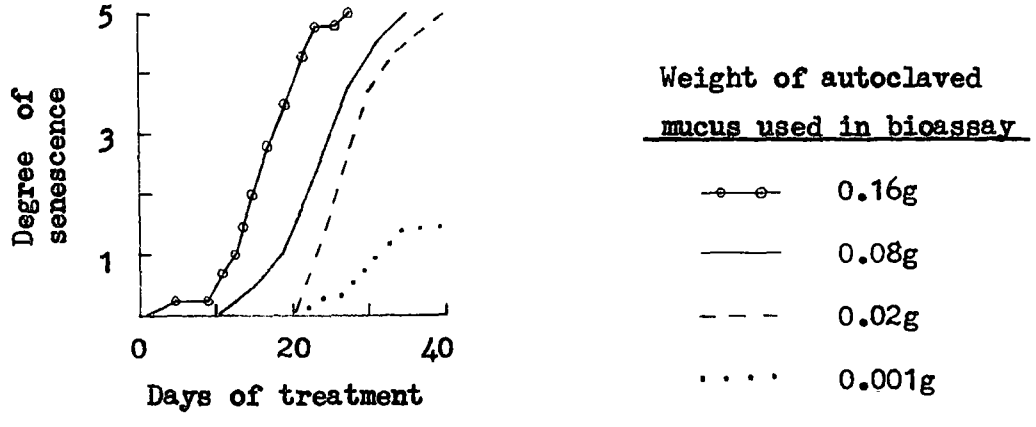


Fig. 38. Response of P.radiata twigs to autoclaved mucus treatment.

Thin layer chromatography (abbreviated TLC)

For the separation of sugars or aminoacids by TLC, cellulose was found to give better results than did silica gel (Kieselguhr). Each individual sugar or aminoacid appeared as a discrete spot with very little diffusion on cellulose plates, whereas these tended to be rather diffused on silica gel plates. The choice of chromatographic solvent was largely dependent on the rates at which the sugars migrated with respect to each other. The R_F's for all of the sugars were low in both BEW (i.e., neutral) and EAW (i.e., acidic) solvents, and although the R_F's were high in n-butanol - acetic - acid (50 : 15 : 35, v/v) there was a tendency for some overlapping of sugar spots. Migration of sugars in the basic solvent benzene - n-butanol - pyridine - water (1 : 5 : 3 : 3, v/v) was very poor as all of the sugar spots formed backward trails. However, the basic solvent BPW was found to be the most satisfactory for the development of hexuronic acids, hexosamines and hexoses on cellulose plates, and it was therefore used in routine TLC. Similarly, the choice of visualization reagents was dependent on the variations of colours produced by closely situated sugar spots, with the reagents. All of the reducing sugars appeared as dark brown spots with alkaline $AgNO_3$, but aniline hydrogen phthalate and aminobiphenyl afforded some degree of colour discrimination and were preferentially employed. The Elson - Morgan reagent was used for the visualization of hexosamines.

Hexuronic acids: they were readily decarboxylated by concentrations of HCl which were greater than 0.2N, for 2hr at 100°C. Both galacturonic and glucuronic acids were detected in the mucus hydrolysates, and they appeared to be present in very low concentrations on the chromato-

gram. The hexuronic acids of S.noctilio mucus reacted with the modified carbazole reagent of Bitter and Muir (1962) to produce a reddish - brown product which had a maximum absorption in the range of 520 - 530nm (fig. 39), and is in agreement with the absorption maximum of 530nm for pure hexuronic acids.

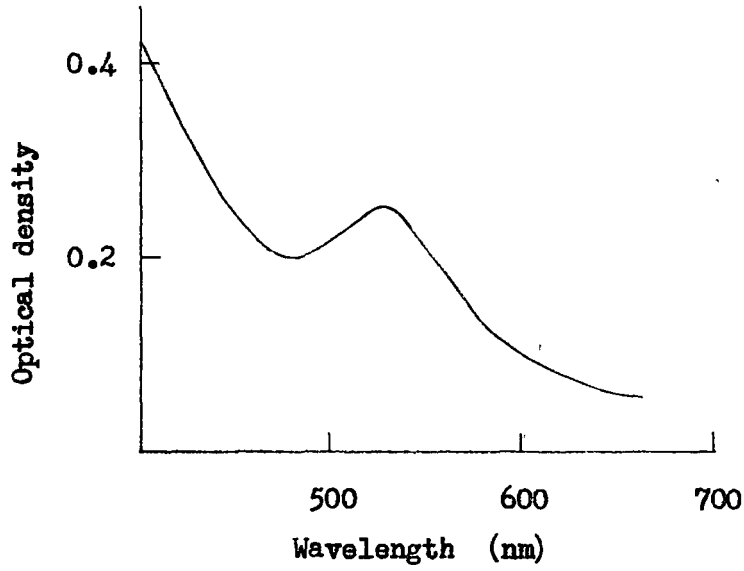
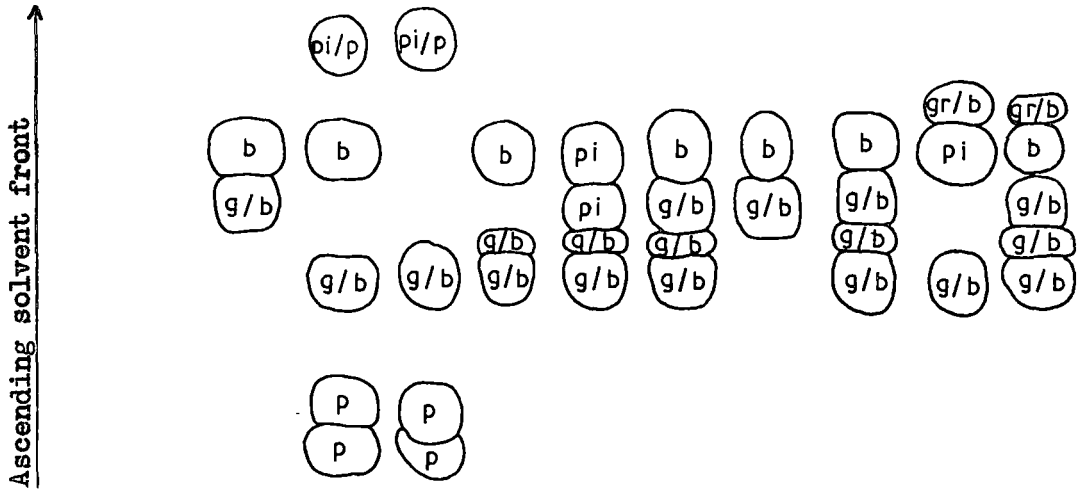


Fig. 39. Absorption spectrum of mucus hexuronic acids in the carbazole reagent.

Hexoses and methylpentoses: these reducing sugars were released by HCl in the concentration range of 0.1 - 8N for 2hr at 100°C (fig. 40). The release of fucose and galactose by low concentrations of HCl (e.g., 0.1N), indicated their terminal or peripheral positions on the mucus molecules. Increasing the concentration of HCl to 0.5N brought about the appearance of glucose; mannose appeared in the 1N HCl hydrolysate. Rhamnose was detected in the 8N HCl hydrolysate, but it is uncertain if rhamnose had arisen out of the conversion from some other reducing sugar, or that it is situated at the core of the molecules. The rela-



Origin: + + + + + + + + + +

Sample: Std_1 M Std_2 M Std_3 M Std_4 M Std_5 M

HCl used. – 0·1 N – 0·5 N – 1 N – 4 N – 8 N

Solvent: B P W Spray reagent: Aminobiphenyl

Standard sugars with the lowest to the highest R_F are:

Std_1 = mannose, fucose

Std_2 = galacturonic acid, glucuronic acid, galactose, glucuronolactone

Std_3 = galactose, glucose, xylose, arabinose

Std_4 = mannose, fucose

Std_5 = galactose, ribose, rhamnose

Abbreviations :

M = mucus

g/b = greenish brown b = brown

pi/p = pinkish purple pi = pink

gr/b = greyish brown p = purple

Fig. 40. Chromatogram showing sugars released from S.noctilio mucus by varying concentrations of HCl, for 2hr at 100°C.

tive concentration of sugars present in a mucus hydrolysate is given in table 16.

Table 16: Visual estimates of colour intensities of sugar spots present in the chromatograms of mucus hydrolysates. Chromatograms for hexoses, hexosamines and hexuronic acids are not directly comparable with each other.

Type of sugar	Relative concentration present
rhamnose	+
fucose	2+
mannose	2+
glucose	+
galactose	2+
glucosamine	+
galactosamine	+
glucuronic acid	+
galacturonic acid	+

Hexosamines: the mucus hexosamines were eluted from Dowex 50 (H^+) into two peaks, F2 and F3 (fig. 41). F2 consisted mainly of glucosamine, but F3 was a mixture of glucosamine and galactosamine. Although F1 gave a positive assay with the Elson - Morgan reagent, it contained no detectable hexosamine on the chromatogram. The mucus hexosamines reacted with the Elson - Morgan reagent to produce a red product with a maximum absorption at 530nm, which coincides with the figure for pure

hexosamines (Boas, 1953), refer to fig. 42.

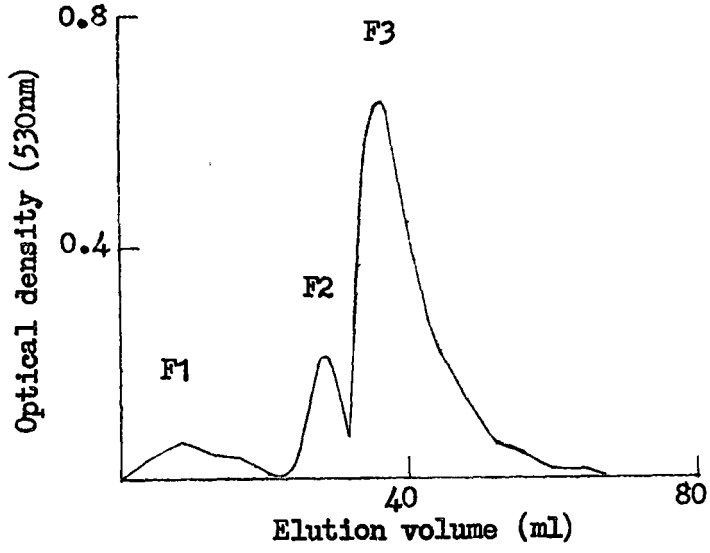


Fig. 41. Elution profile of mucus hexosamines from Dowex 50 (H^+).

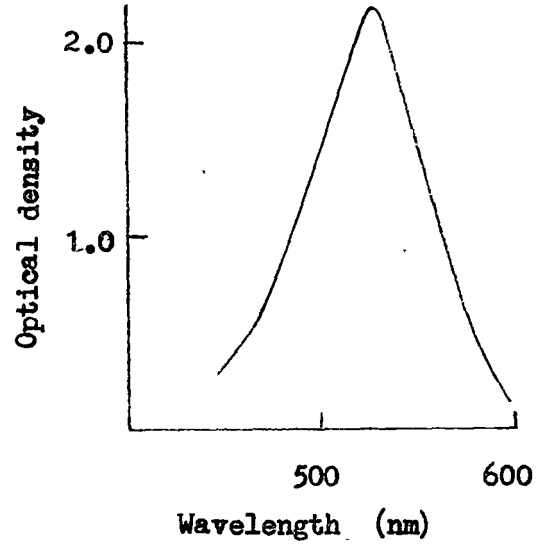


Fig. 42. Absorption spectrum of mucus hexosamines in the Elson - Morgan reagent.

During chromatographic separation, the interference of salt crystals present in the dried residues of the acid eluates caused streakiness to occur, so that it was not possible to differentiate between glucosamine and galactosamine. The problem was not overcome by selective solution of hexosamines in dry pyridine, as a certain amount of salt was dissolved in pyridine whilst some hexosamines remained with the salt crystals in the pyridine insoluble fraction (fig. 43). As a result, it was necessary to first subject the mucus hexosamines to ninhydrin degradation and then identify the pentose derivatives formed from this reaction. Glucosamine and galactosamine were detected in this way (fig. 43).

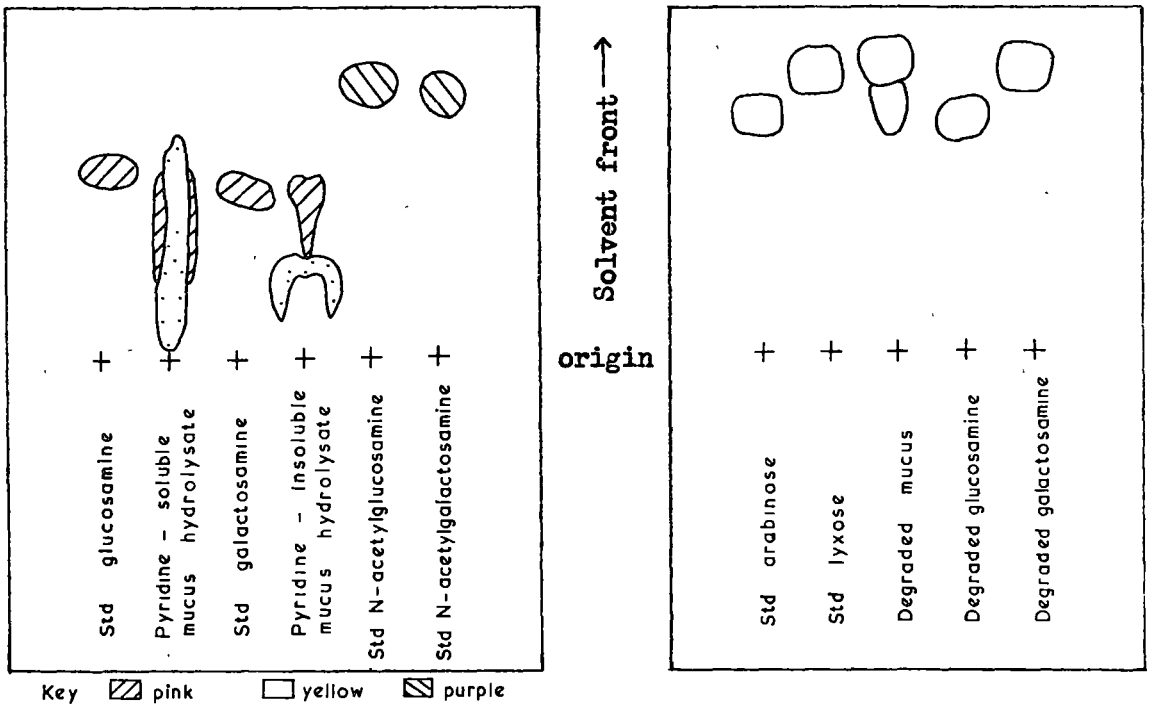


Fig. 43. Chromatograms of hexosamines (A), and their pentose derivatives (B) following ninhydrin degradation.

Chromatogram A: Spray reagent = Elson - Morgan reagent.
Chromatogram B: Spray reagent = aniline hydrogen phthalate.

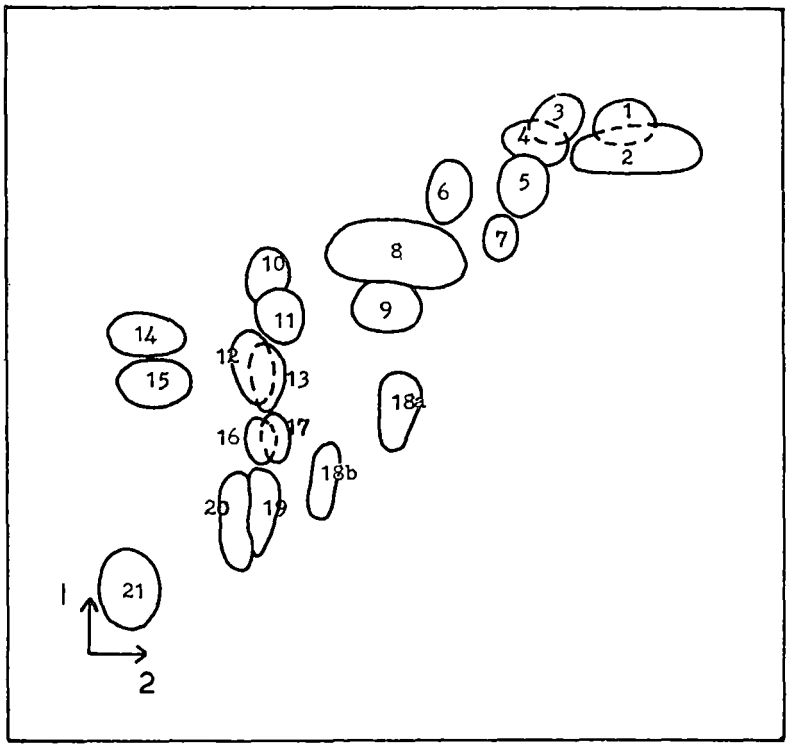
Aminoacids: a total of 17 aminoacids were identified by TLC, and typical chromatograms of both authentic aminoacids and of the mucus hydrolysate are given in fig. 44. The identification of individual aminoacids is dependent on its position on the chromatogram with respect to two adjacent solvent fronts, and on the colour reaction with ninhydrin. Although spot 16 on chromatogram B did not correspond in relative position with spot 16 (i.e., glutamine) on chromatogram A, it was nevertheless identified as glutamine. This was confirmed on mixing authentic glutamine with the mucus hydrolysate, and when both authentic glutamine and mucus spot 16 appeared as a single entity on the chromatogram.

Sulphate

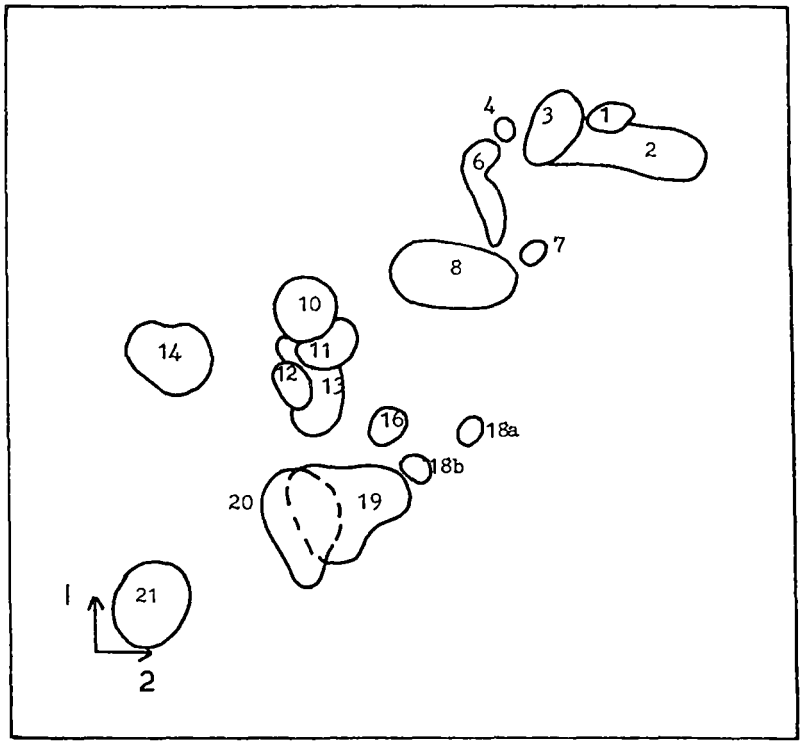
In earlier tests using basic lead acetate and $FeCl_3$, both fresh and aged mucus solutions gave negative results for -SH groups (table 3). The reaction of S.noctilio mucus in toluidine blue was orthochromatic and was typical of the reactions given by unsulphated mucopolysaccharides (pages 200 to 203).

A mucus hydrolysate (50ug/ml of 1N HCl, at 100°C overnight in a sealed ampoule) was found to produce a fine white precipitate with $BaCl_2$, which indicated the presence of sulphate in the mucus (Boros, 1968). On applying the same test to a mucus solution which had been treated with HCl (20mg mucus in 1ml of water, was acidified with HCl and heated) no precipitate was detected, and Gaut (1970) concluded that sulphate was not present in significant quantities and also suggested that Boros' sample might have been "contaminated from an extraneous source". In my own work using two different tests, based on the turbidimetric method of Dodgson and Price (1962) and the spectrophotometric method

Fig. 44. Chromatograms of authentic aminoacids (A), and of S.noctilio mucus aminoacids (B).



A: Chromatogram of authentic aminoacids



B: Chromatogram of mucus hydrolysate

KEY

1. phe (orange)
2. thr (blue)
3. leu (purple)
4. trp (brown)
5. met (purple)
6. val (purple)
7. tyr (brown)
8. ser (grey-brown)
9. his (pink)
10. pro (yellow)
11. ala (purple)
12. gly (mushroom-brown)
13. hyp (orange)
14. lys (purple)
15. orn (purple)
16. glu(NH_2) (purple)
17. asp(NH_2) (orange)
18a. cysteic acid (purple)
18b. cys (mauve)
19. glu (purple)
20. asp (sky-blue)
21. arg (purple)

1 → 2 indicates direction of solvent fronts

Solvent systems: I/II

of Iwasaki, et al (1957), sulphate was detected in low concentrations in the mucus hydrolysate (tables 17 and 18).

Table 17: Results of sulphate analysis on a mucus hydrolysate, based on the turbidimetric method of Dodgson and Price (1962).

Sample	O.D. at 360nm				∴ O.D. due to SO_4^{2-}
	A	B	C	D	
1.	0.074	0.014	0.050	0.00	0.010
2.	0.488	0.294	0.180	0.00	0.014

A = test solution consisting of mucus hydrolysate, $BaCl_2$ and gelatin.

B = control solution consisting of mucus hydrolysate and gelatin.

C = blank solution consisting of $BaCl_2$ and gelatin.

D = blank solution consisting of gelatin alone, and was used as the reference solution against which all other samples were compared.

O.D. due to mucus sulphate = A - B - C - D.

An average O.D. of 0.012 is equivalent to 0.0001N H_2SO_4, in 0.2g of fresh mucus.

Table 18: Results of sulphate analysis on a mucus hydrolysate, using the spectrophotometric method of Iwasaki, et al (1957).

Sample	O.D. at 370nm		∴ O.D. due to SO_4^{2-}
	Mucus + Reagent	Mucus - Reagent	
1.	1.28	0	1.28
2.	1.20	0	1.20
3.	1.30	0	1.30

An average O.D. of 1.26 is equivalent to 0.0068% K_2SO_4.

The concentration of sulphate present in 1g of fresh mucus was calculated at 6.8mg/ml (based on K_2SO_4 calibration). This is a very low concentration, and its presence in S.noctilio mucus had been confirmed by two different analytical procedures. An earlier publication by Wong and Crowden (1976) reported on the absence of sulphate in S.noctilio mucus. The data used then, were derived from tests using very dilute mucus hydrolysate solutions (about ten times more dilute) which resulted in negative readings. It is felt that a similar negative result obtained by Gaut (1970) was probably due to incomplete hydrolysis of the mucus.

Sialic acid

These monosaccharides are highly acidic and occur as prosthetic groups with glycoproteins, glycopeptides and glycolipids. They are of common occurence in vertebrates where they confer high viscosities to the glycoproteins or glycolipids, and are therefore of vital importance physiologically. Their occurence in invertebrates is sporadic and uncertain, and so far, they have been detected in certain species of Turbellaria and Trematoda (phylum Platyhelminth), Gastropoda and Cephalopoda (phylum Mollusca), Crustacea (phylum Arthropoda), Asteroidea, Ophiuroidea, Echinoidea, Holothuroidea and Crinoidea (phylum Echinoderma), Hemichorda and Cephalochorda (phylum Chordata). In S.noctilio mucus, a series of experiments seem to indicate the absence of sialic acid. Results of these experiments are as follows:

a) A crude mucus solution on being hydrolysed with dilute H_2SO_4 gave rise to a steady increase of material which reacted with the Ehrlich reagent to form a blue coloured product. The rate of release of this reactant is shown in fig. 45a.

b) A 0.5% solution of autoclaved mucus gave a reading of O.D. = 0.23 at 565nm with the Ehrlich reagent, although authentic samples of monosaccharides (i.e., glucose, galactose, mannose, fucose, rhamnose, glucosamine and galactosamine) did not produce any coloured reactions with the same reagent. This seemed to indicate the probable presence of "free" sialic acid in autoclaved mucus. However on determining the absorption spectrum of a partially purified solution of autoclaved mucus (after its passage through AG1 X2, Ac^- form) in the Ehrlich reagent, a broad band of absorption from 570nm to 640nm was recorded (fig. 45b). These figures do not agree with the absorption maxima of 530nm and 565nm for sialic acid (Werner and Odin, 1952). It is apparent that some substance other than sialic acid was responsible for the coloured reaction with the Ehrlich reagent.

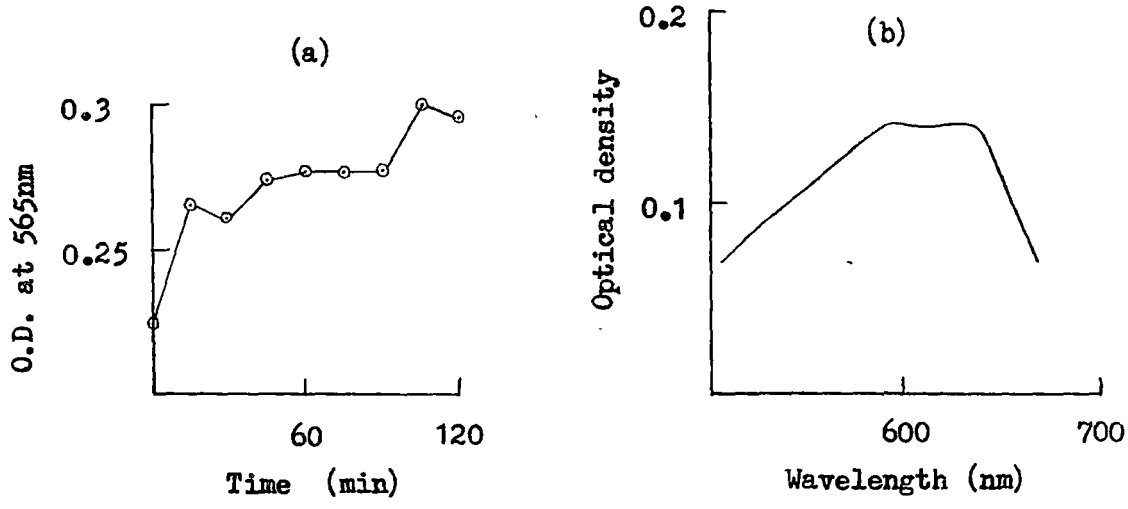


Fig. 45. (a) Rate of release of Ehrlich positive material from S.noctilio mucus during its digestion with dilute H_2SO_4.

(b) Absorption spectrum of the product obtained from reacting a partially purified autoclaved mucus sample with the Ehrlich reagent.

c) Using the thiobarbituric acid method of Warren (1959) for the determination of sialic acid, both acid - hydrolysed and unhydrolysed mucus solutions reacted with the reagents to produce an orange coloured product with maximum absorptions at 450nm and 523nm (fig. 46). These figures are not in agreement with the absorption maximum of 549nm for pure sialic acid. Thus, on the basis of two different assay procedures, S.noctilio mucus does not appear to contain sialic acid.

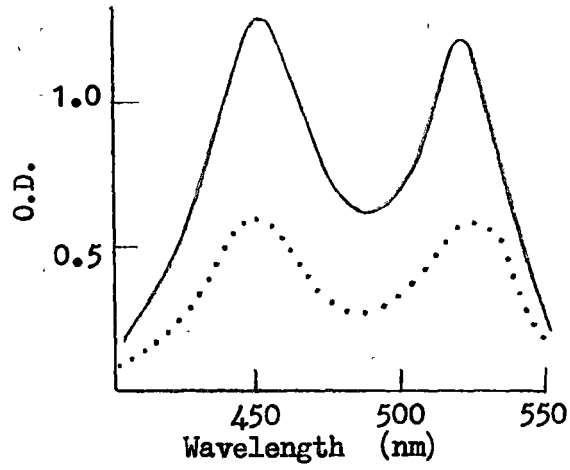


Fig. 46. Absorption spectrum of autoclaved mucus in the thiobarbituric acid reagent.

——— autoclaved mucus

····· acid - hydrolysed autoclaved mucus

Electrophoresis on a preparative scale

The relationship between protein concentration and electrophoretic banding pattern for an aged mucus solution is given in fig. 47. On its own, the blank gel was found to be quite reactive with the rea-

gents of Lowry, et al (1951).

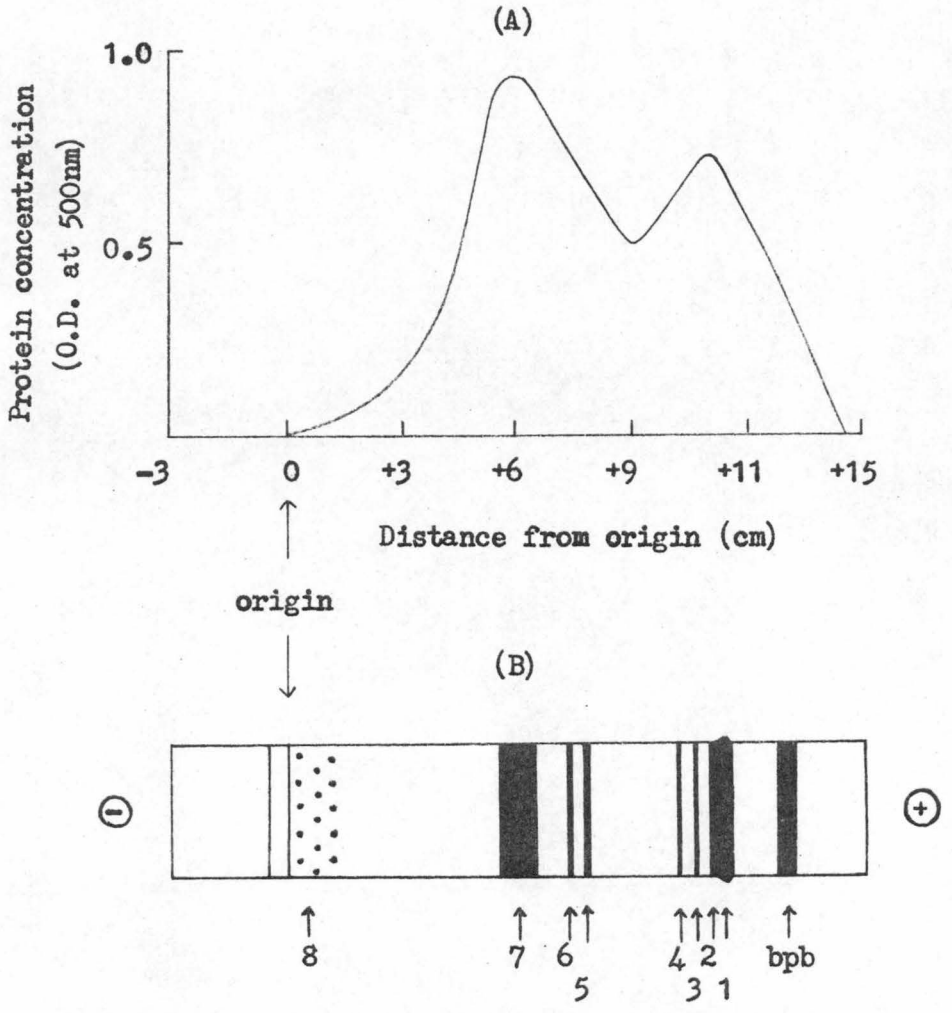


Fig. 47. Preparative electrophoresis of an aged mucus solution, showing the concentration of protein extracted from each strip of gel (A), and the corresponding protein banding pattern (B). Bromophenol blue (bpb) was used as the marker dye.

Several limitations have to be overcome before this very useful technique can be used for the isolation of individual protein bands

for chemical analyses and for bioassays. For the start, a high voltage power source is required to reduce the present period of 18hr of electrophoretic separation (the maximum capacity of the laboratory power pack is 240volts). Recovery of S.noctilio mucus from the acrylamide gel by suction filtration was very slow and tedious, but for future work, the protein bands may be eluted from the gel by application of an electric current. The close proximity of the fast migrating protein bands make their individual isolation by the present methods rather awkward, and it is not intended to further pursue this technique of isolation until such time when funds are available for the purchase of high voltage electrophoresis equipment, or when more time is available to improve upon the technique with the existing resources.

Gel filtration

a. Raw mucus.

The protein and carbohydrate fractions of raw mucus were eluted almost in parallel from Biogel P60, and they were predominantly in the void volume (fig. 48). This indicates the presence of material whose molecular weight is greater than 60,000. The attenuated elution profile of aged mucus suggests that some smaller molecules (of molecular weight less than 60,000) were retained in the gel column (fig. 48c). An unsuccessful attempt at eluting mucus through Biogel P100, due to "gumming - up" of the column by adsorption of mucus, suggests the absence of molecules which are greater than 100,000 in molecular weight.

Preparation of raw mucus in NaCl solution resulted in some disaggregation of the high molecular weight components and a displacement of the elution profile (fig. 48b). As with aged mucus, retention of smaller molecules by the gel column was also apparent. In spite of the

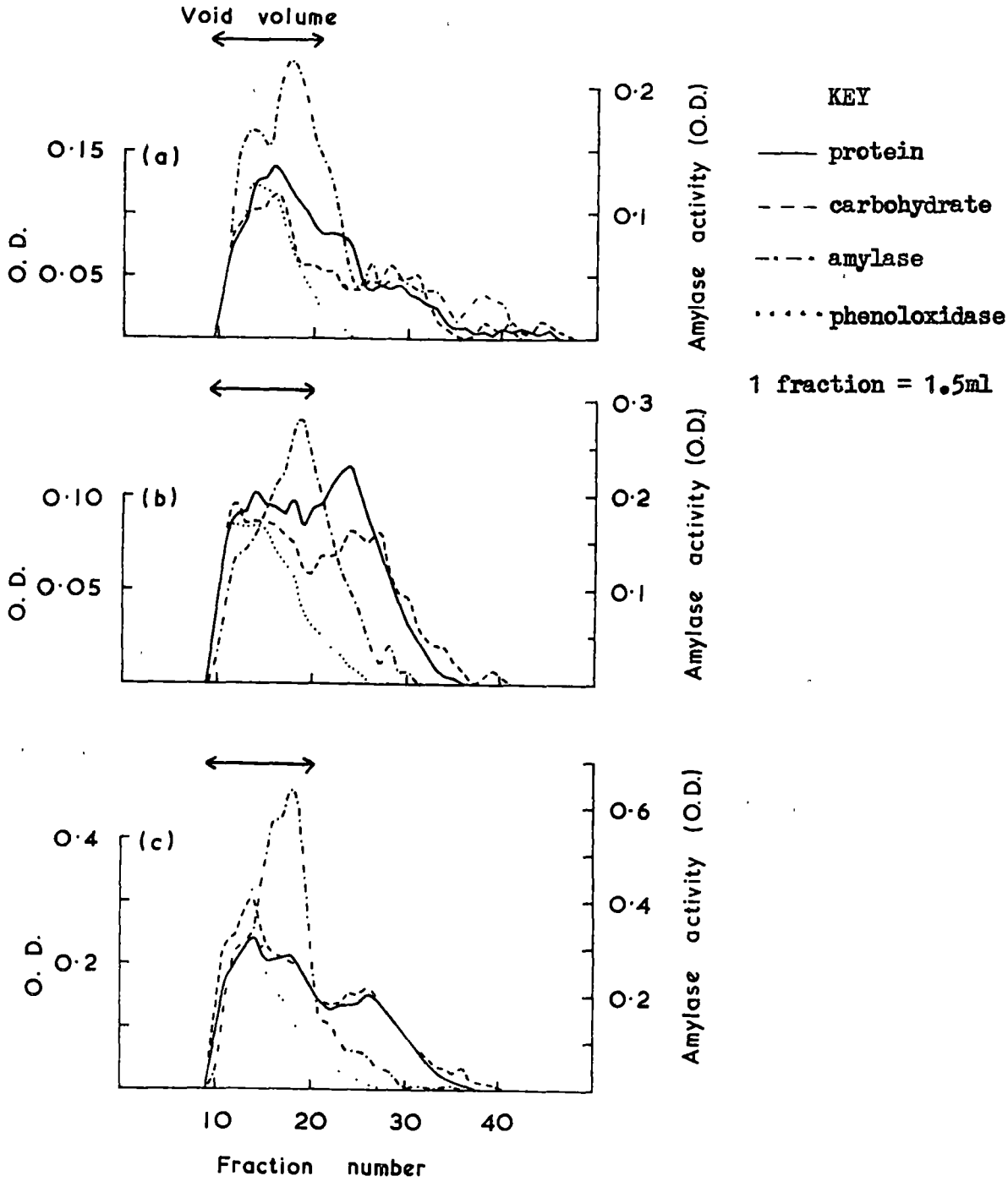


Fig. 48. Elution profiles of S.noctilio mucus (4% solution, stored at 2°C), from Biogel P60 at 2°C.

(a) 1 week old mucus solution, using 2ml.

(b) 1 week old mucus solution in the presence of 1% NaCl, using 2ml.

(c) 18 week old mucus solution, using 4ml.

The scale on the left vertical axis refers to optical densities of protein and carbohydrate, and activities of phenoloxidase.

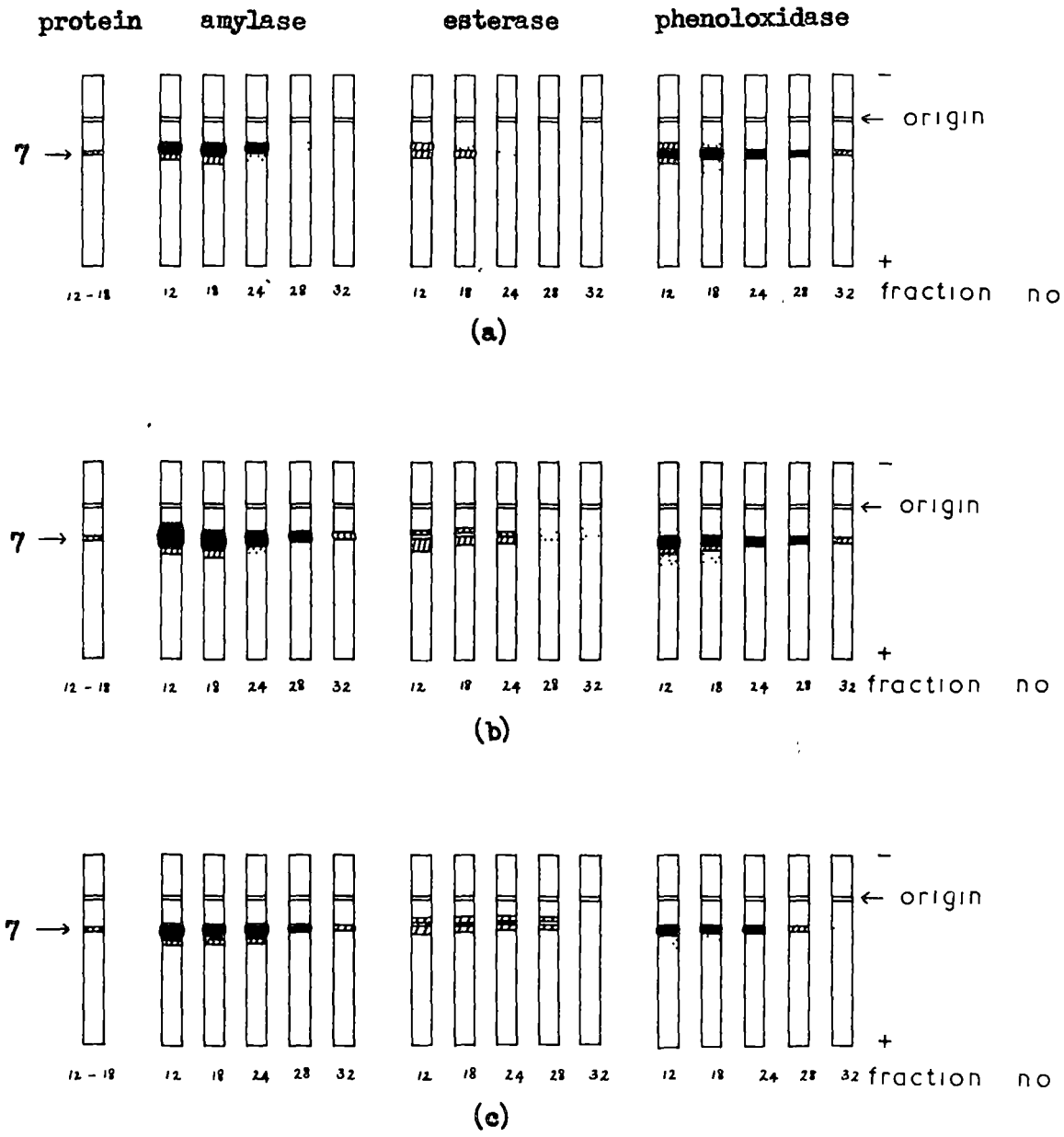


Fig. 49. Electrophoretograms of elution fractions from Biogel P60.

(a) 1 week old mucus.

(b) 1 week old mucus in the presence of 1% NaCl.

(c) 18 week old mucus.

The single protein band present is equivalent to band number 7 of figure 53a.

observed alterations to the elution patterns of protein and carbohydrate, the amylase and oxidase enzymes did not show displacements in their elution profiles as they were essentially associated with the high molecular weight components which eluted in the void volume of P60.

Electrophoretograms of eluant fractions from P60 indicated the presence of a single protein zone, derived mainly from the void volume fractions. Fractions from the elution volume (i.e., beyond fraction 20) did not appear to stain up with amido black. Amylase and esterase were present as two closely associated isoenzymic bands whereas phenoloxidase appeared as a single band, located at or near the position of protein band number 7 (fig. 49). The elution profiles of amylase and phenoloxidase broadly confirmed their electrophoretic patterns. Other protein bands of different electrophoretic mobilities were not detected.

b. Raw mucus, following treatment with cold acetone.

Raw mucus was precipitated with cold 80% acetone as a white substance which redissolved in deionised water with less readiness than the untreated mucus. Its elution from Biogel P60 gave rise to two major well - defined carbohydrate peaks, and three protein peaks of which the first two were closely associated (fig. 50b). Phenoloxidase was mainly associated with the first protein peak and amylase was associated with the second and third protein peaks. Physiological activities were detected in both the void and elution volumes, although most of the activity was confined to fractions 21 - 33 which had molecular weights of below 60,000.

A solution of mucus precipitate, after 18 weeks of storage at 2°C, showed an attenuated elution profile beyond the void volume (fig. 50a). Both protein and carbohydrate were eluted in parallel. The physiologi-

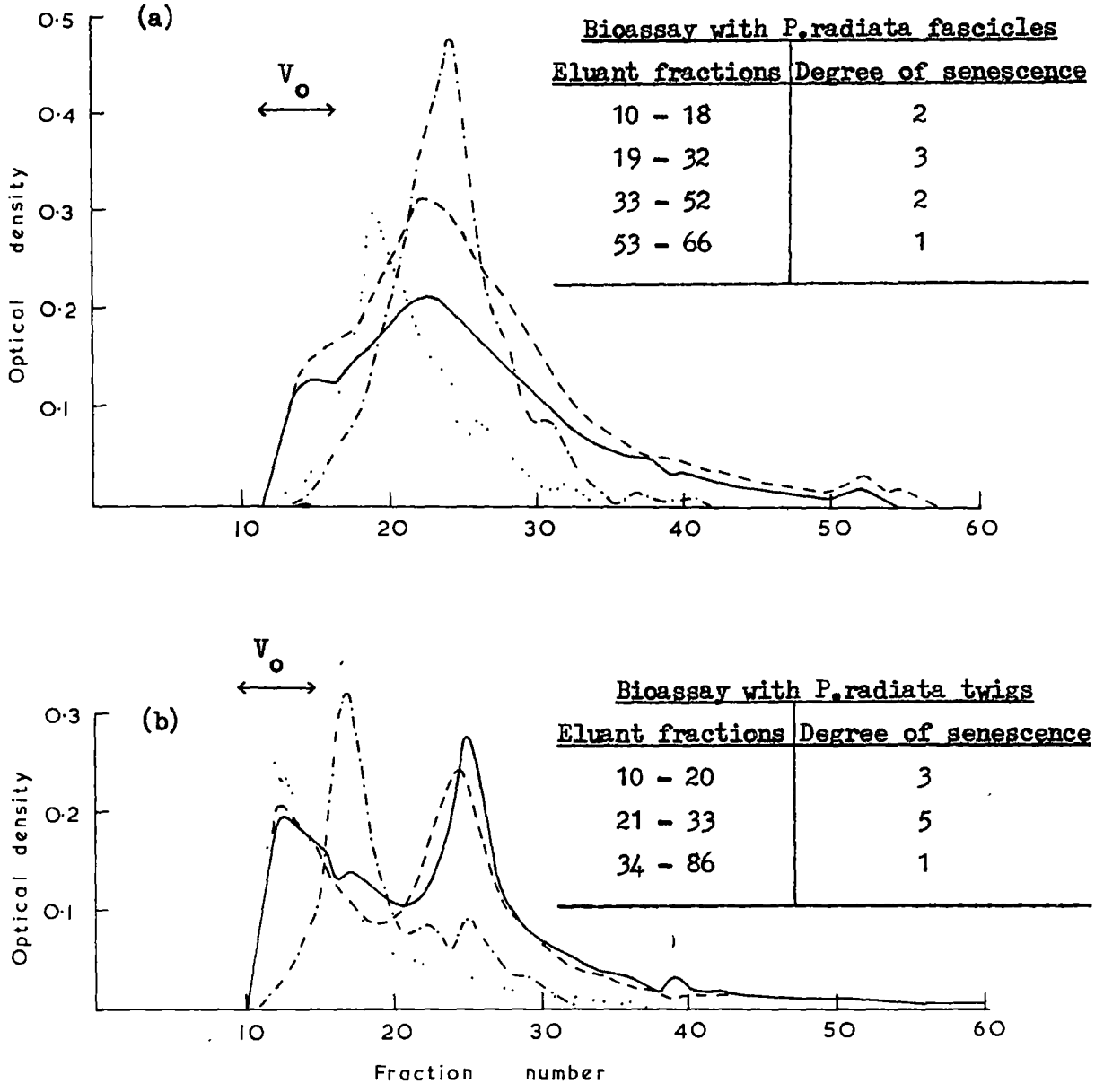


Fig. 50. Elution profiles of acetone - treated mucus (4ml of 4% solution) from Biogels at 2°C.

(a) aged mucus (stored at 2°C for 18 weeks) from Biogel P30.

(b) fresh mucus from Biogel P60.

——— protein -·-·- amylase

- - - - carbohydrate · · · · phenoloxidase

V_o = void volume

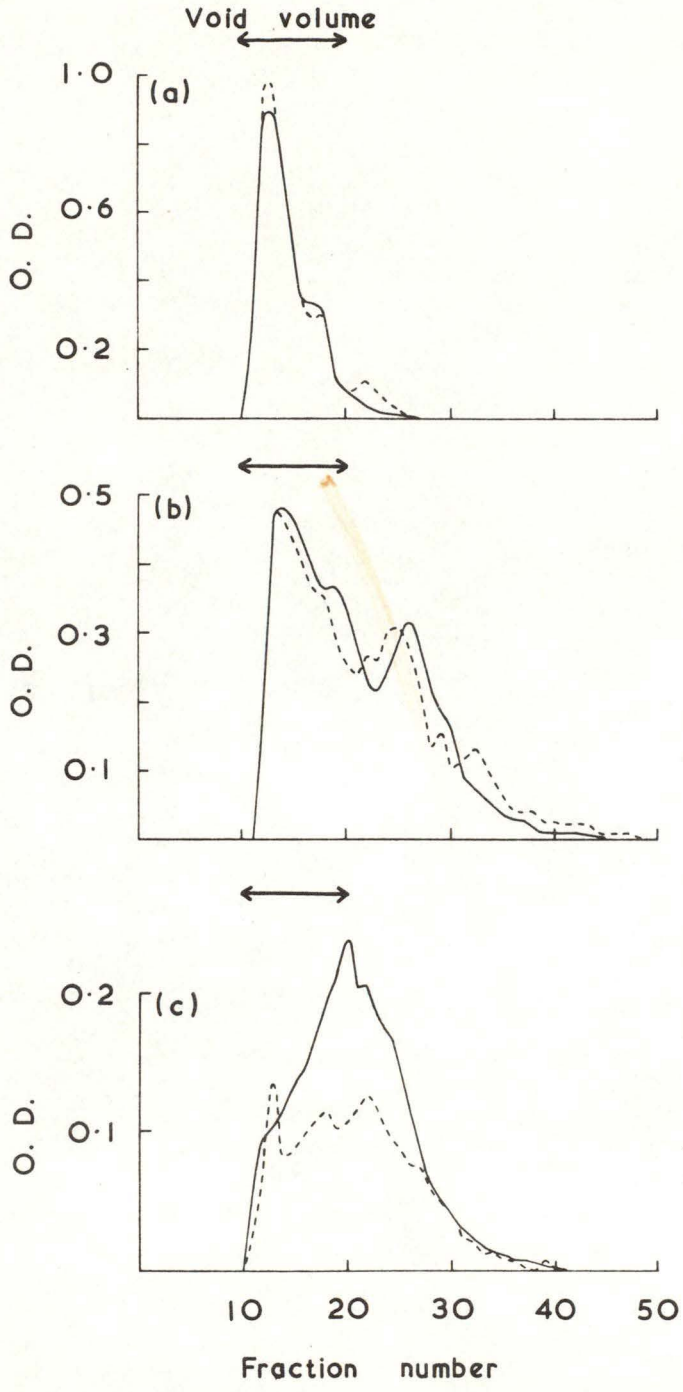
cal activity was apparently retained to some extent by the P30 Biogel, as fractions 53 - 66 which contained immeasurable amounts of protein and carbohydrate had weak physiological activity. These physiologically active molecules, smaller than 30,000 in molecular weight, may have arisen from spontaneous disaggregation of the macromolecule through prolonged aging.

c. Autoclaved mucus.

Extensive disaggregation of raw mucus by autoclaving produced a solution of relatively low viscosity (fig. 33a). On the basis of filtration through Biogels P2, P6 and P10, its molecular weight was estimated to be predominantly in the order of less than 10,000 (fig. 51). Molecules larger than 10,000 in molecular weight were also present, but as the autoclaved mucus solution failed to fractionate from Biogel P30, it is assumed that none of the molecules were of a molecular weight of 30,000.

A slight displacement in the elution profiles of protein and carbohydrate were noted in the Biogel P6 fractionation; and whilst essentially eluting in parallel, there appeared to be a higher concentration of protein (compared with carbohydrate) in the P10 eluants. It is suggested that the unmasking of reactive sites (refer to tests 7, 8 and 11 of table 3) is probably the cause of the apparently higher protein concentration.

The destruction of all enzymatic activities by autoclaving was not accompanied by the loss of physiological activity from the mucus solutions. The eluant fractions of P2 were most active in inducing P.radiata twig senescence, but the eluant fractions of P10 were comparatively less active (fig. 52), and is due to a higher retention of



(a) using Biogel P2 (4ml of 4% AM).

(b) using Biogel P6 (4ml of 4% AM).

(c) using Biogel P10 (4ml of 4% AM).

Fig. 51. Elution profiles of autoclaved mucus from Biogels, at room temperature. Each fraction consists of 1.5ml.

—— protein — — — carbohydrate

the physiologically active fractions by Biogel P10. More physiologically active molecules were also extracted from the P10 than from the P6 column residues, but exhaustive extraction of the P2 column failed to yield any active fractions beyond fraction 25 (fig. 51a), indicating that the lower limit for retention of physiological activity by the glycopeptides was about molecular weight 2,000.

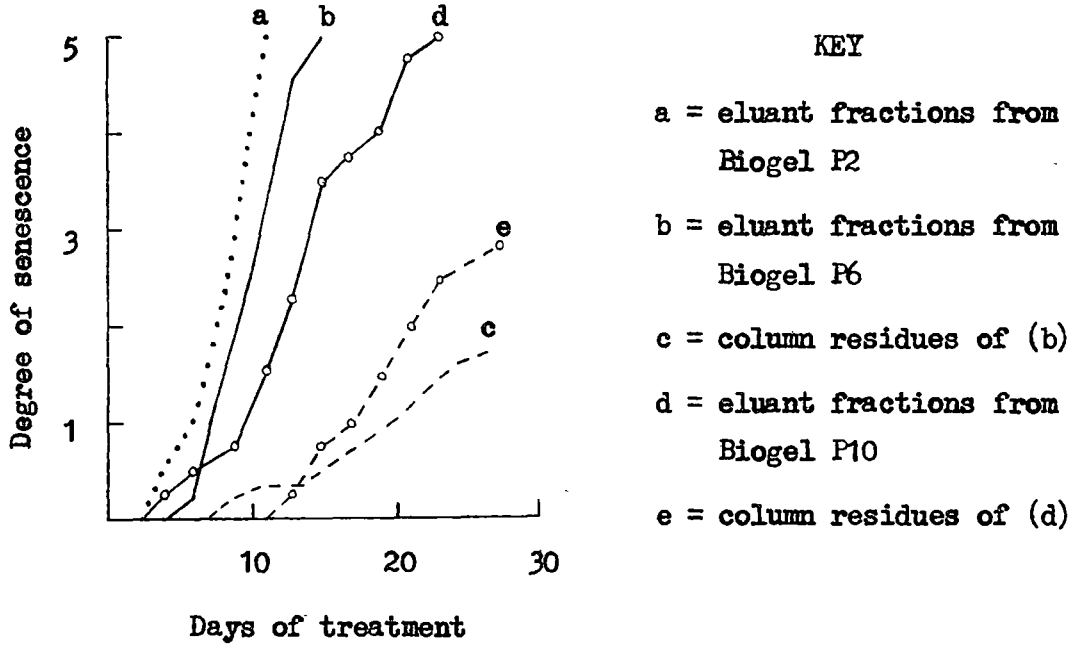
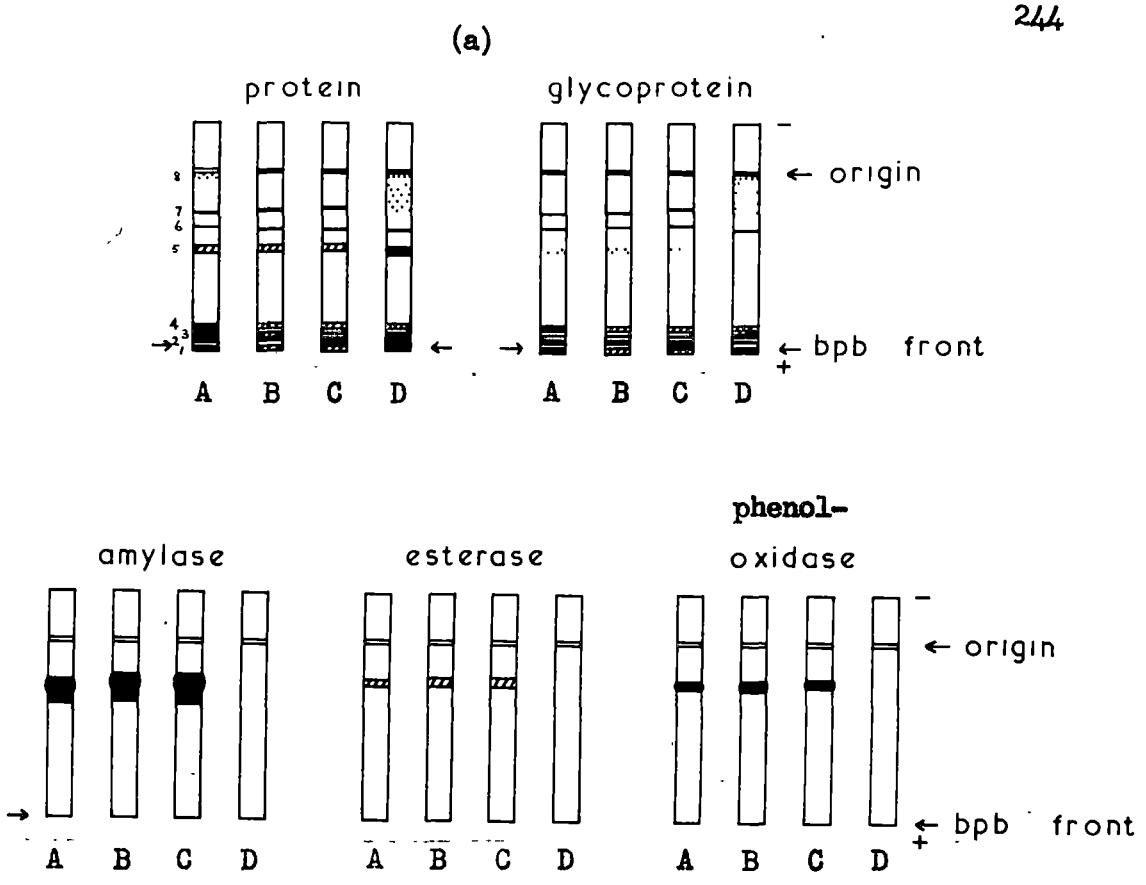


Fig. 52. Bioassay of Biogel eluates and column residues of autoclaved mucus, with P.radiata twigs.

Electrophoretograms of some of the P10 eluant fractions, together with a sample of unfractionated autoclaved mucus, are shown diagrammatically in fig. 53b. Variations in intensity of staining between different electrophoretograms, of some of the weaker protein and glycoprotein bands (numbers 5, 6 and 7) of unfractionated autoclaved mucus are apparent (compare (a)D and (b)AM of fig. 53). The void volume fractions of Biogel P10, fractions 13 and 15, consist of protein bands number 8



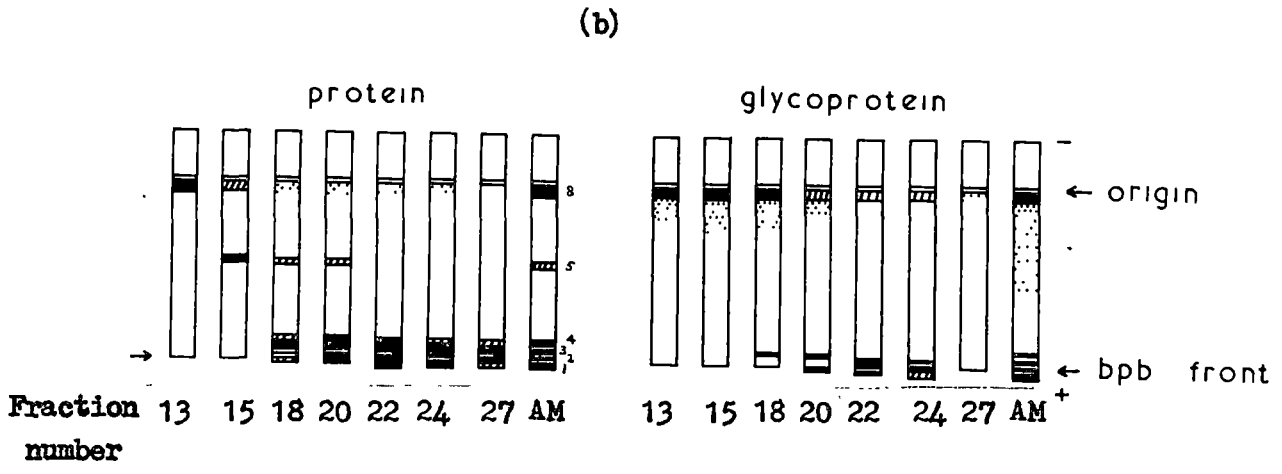


Fig. 53. Electrophoretograms of S.noctilio mucus.

(a) effects of inorganic salts or autoclaving.
A = raw mucus solution
B = raw mucus in the presence of 0.5% NaCl
C = raw mucus in the presence of 0.2% $CaCl_2$
D = autoclaved mucus solution

(b) eluant fractions of autoclaved mucus from Biogel P10, derived from the fractionation of figure 51c.
AM = sample of unfractionated, autoclaved mucus.

and 5, respectively, and are therefore in the molecular weight range of 10,000 - 30,000. Thereafter, was the elution in strength of high electrophoretic - mobility protein bands from fraction 18 onwards. As these bands appeared in the void volume of Biogel P6, they may be assumed to be of molecular weight 5,000 to 10,000. Protein band number 7, which normally contains enzymatic activities, had disappeared from autoclaved mucus.

All of the protein bands were reactive with the P.A.S. reagent, but with low intensities of staining. They also stained purple with toluidine blue, blue - green with alcian blue, and red with acridine orange.

d. Aged mucus.

Elution profiles from Biogel P6, of aged mucus solutions (i.e., aged for 125hr and 250hr, at 37°C) were different from that of autoclaved mucus (compare figs. 54 and 51b). A significant proportion of mucus eluted after the void volume (therefore, of molecular weight less than 5,000), in the 250hr sample. As with autoclaved mucus, the elution patterns of protein and carbohydrate of these aged mucus samples were not in parallel. These elution patterns generally confirm earlier observations on the increasing proportion of smaller molecules in the aged samples, produced as a result of disaggregation of the macromolecule. The electrophoretic patterns of these aged mucus samples have been illustrated in figure 35, and their physiological activities indicated in table 15.

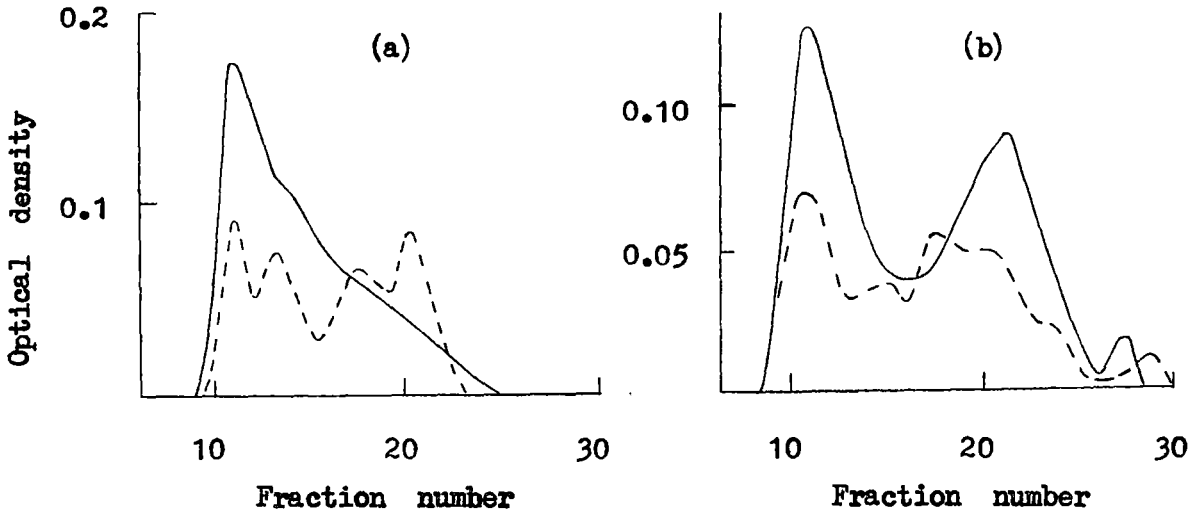


Fig. 54. Effect of aging on the elution profile of S.noctilio mucus (0.05g mucus/2ml H_2O), from Biogel P6.

(a) mucus stored at 37°C for 125hr.

(b) mucus stored at 37°C for 250hr.

e. Mucus, following treatment with 40% aqueous phenol at 60°C.

Raw mucus was precipitated from solution, in the presence of phenol and 0.2M NaCl. Aqueous solutions of the water, phenol and interphase (i.e., mucus precipitate) layers were found to contain protein and carbohydrate, as detected with tests 2 and 5, of table 3. Each of these three extracts produced nine monosaccharides on acid hydrolysis. The relative proportion of each monosaccharide in the three extracts, is given in table 19.

On the basis of sugar analysis, it appears that S.noctilio mucus is soluble in phenol, to a limited extent. It was not possible to isolate any polysaccharide fraction which was free from protein. However, using this same method, Hunt and Jevons (1963) had isolated glycopro-

teins in the interphase layer, from the hypobranchial mucin of Buccinum undatum. The water extract of hypobranchial mucin consisted of protein - free polysaccharides, whilst the phenol extract consisted of a very small proportion of protein.

Table 19: Visual estimates of relative sugar concentrations, in the acid hydrolysates of water, phenol and interphase extracts of S.noctilio mucus.

	Relative concentration in each extract		
Sugars	Interphase	Aqueous	Phenol
rhamnose	+	+	+
fucose	3+	+	+
mannose	2+	+	+
glucose	+	+	+
galactose	3+	+	+
glucosamine	+	+	+
galactosamine	+	+	+
glucuronic acid	+	trace	trace
galacturonic acid	+	trace	trace

Mucus derived from phenol precipitation was autoclaved, and fractionated on Biogel P6. Its elution profile was practically similar to that of an untreated autoclaved mucus solution (compare figs. 55a and 51b), and the physiological activities of both solutions were also similar. Electrophoretograms of the two samples (fig. 55b) were found to

contain similar glycoprotein banding patterns. Thus, phenol may be used as a means of "purifying" the mucus, in the same manner in which acetone is generally employed for the precipitation of proteinaceous material.

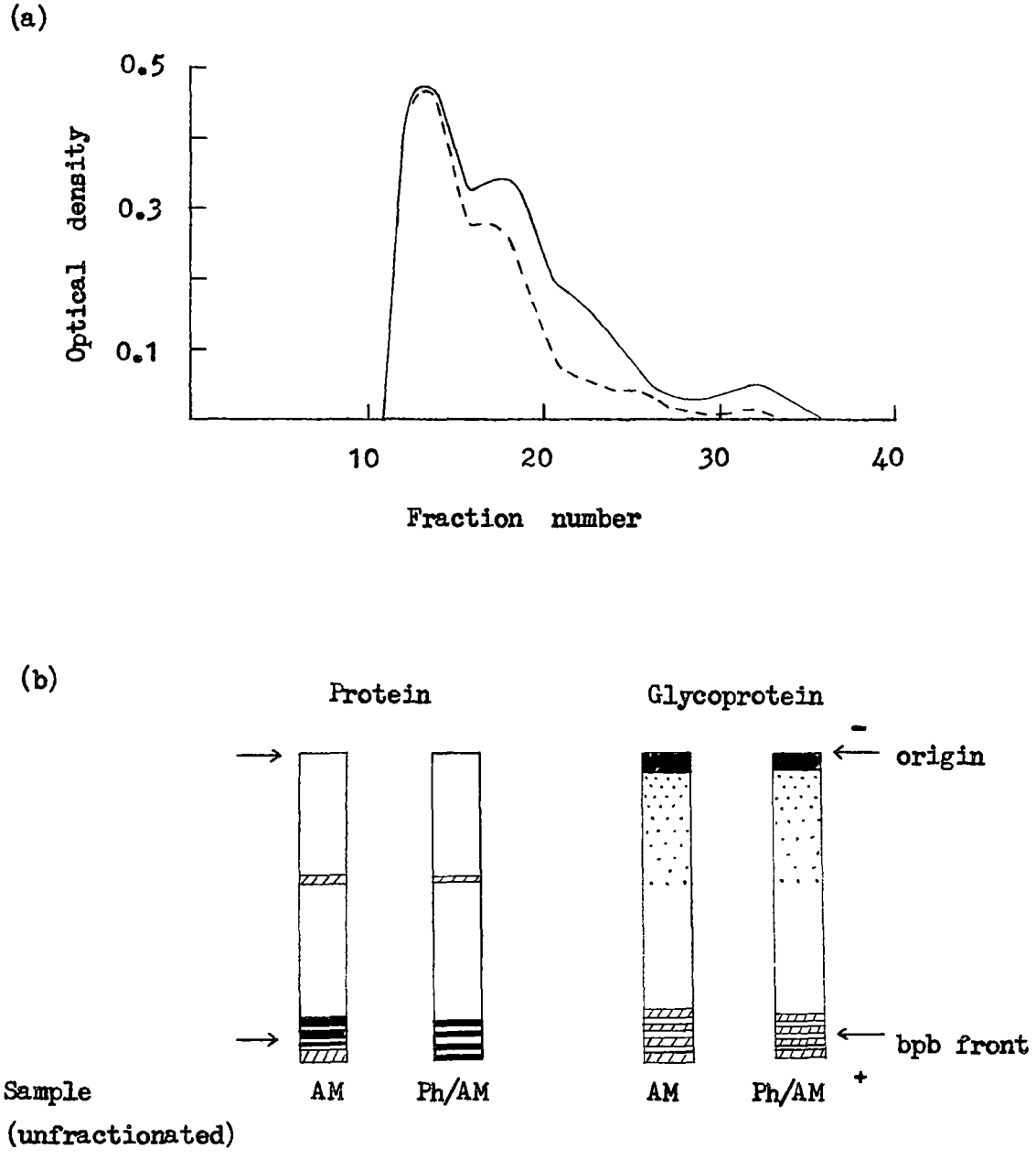


Fig. 55. Elution profile (a) and electrophoretograms (b) of autoclaved phenol - precipitates of S.noctilio mucus.

Ph/AM = autoclaved phenol - precipitates of mucus

f. Enzymatic digestion.

(i) effect of trypsin on raw mucus.

On comparing the elution profile of trypsinated mucus (fig. 56a) with that of untreated raw mucus (fig. 48a), it is apparent that a certain amount of enzymatic breakdown (i.e., with trypsin) had resulted in the formation of a high proportion of smaller molecules which eluted after the void volume of Biogel P60. There was a resistant core of mucus protein which was not affected by trypsin; these were eluted in the void volume of Biogel P60, and were associated with amylase and phenoloxidase. The physiological activity of raw mucus was not apparently affected by trypsin.

The eluant fractions which were subjected to electrophoresis, did not appear to stain with amido black for protein. However, enzymatic activities were located at or near the region of protein band number 7. Additional esterase and phenoloxidase bands were located on the electrophoretograms. These new isoenzymatic bands, "b" for esterase and "a" and "b" for phenoloxidase, were apparently derived from treatment of mucus with trypsin, as they were not observed in previous electrophoretograms of either fresh or aged mucus (refer to fig. 49 (a) and (c)).

(ii) effect of trypsin, papain and cellulase, on autoclaved mucus.

The elution profiles of these enzymatically - treated autoclaved mucus samples, from Biogel P6, showed the existence of a resistant core of protein and carbohydrate (figs. 57, 58 and 60), which was located as a wide band close to the origin of the electrophoretogram (fig. 59). Additional protein bands of high electrophoretic mobility were present but bands 5, 6 and 7 had disappeared from the papain - treated

sample. In the absence of more precise information on the characteristics of these highly mobile bands, they have been assigned a different nomenclature system from that of the control sample which had not been subjected to enzymatic treatment (thus, compare fig. 59 a and b).

The different effects of trypsin, papain and cellulase on autoclaved mucus, gave rise to differing elution profiles (figs. 57, 58, and 60) but each enzymic digest had its own characteristic elution pattern. Prolonged treatment with cellulase had not caused the disappearance of molecules larger than 10,000 in molecular weight (compare figs. 61 (a) and (b), with fig. 51c).

There was a tendency for a reduction in physiological activities of the eluant fractions with a corresponding increase in physiological activities of the glycopeptides in the column residues, of trypsin treated samples (table 20). Prolonged treatment with papain led to a slight reduction in the physiological activities of both the eluant fractions and column residues (table 20), whereas treatment with cellulase tended to improve the physiological activities of the eluant fractions when compared with the untreated samples, but at the same time caused a reduction in the physiological activities of the column residues (table 21).

The specific actions of trypsin, papain and cellulase have not yet been investigated. The aim of this exercise in the first instance, was to determine if the autoclaved mucus was susceptible to enzymatic breakdown, and the extent to which any of the breakdown products retained physiological activities. A future programme could concern itself with investigations of the nature of the fragments which result from enzymatic degradation.

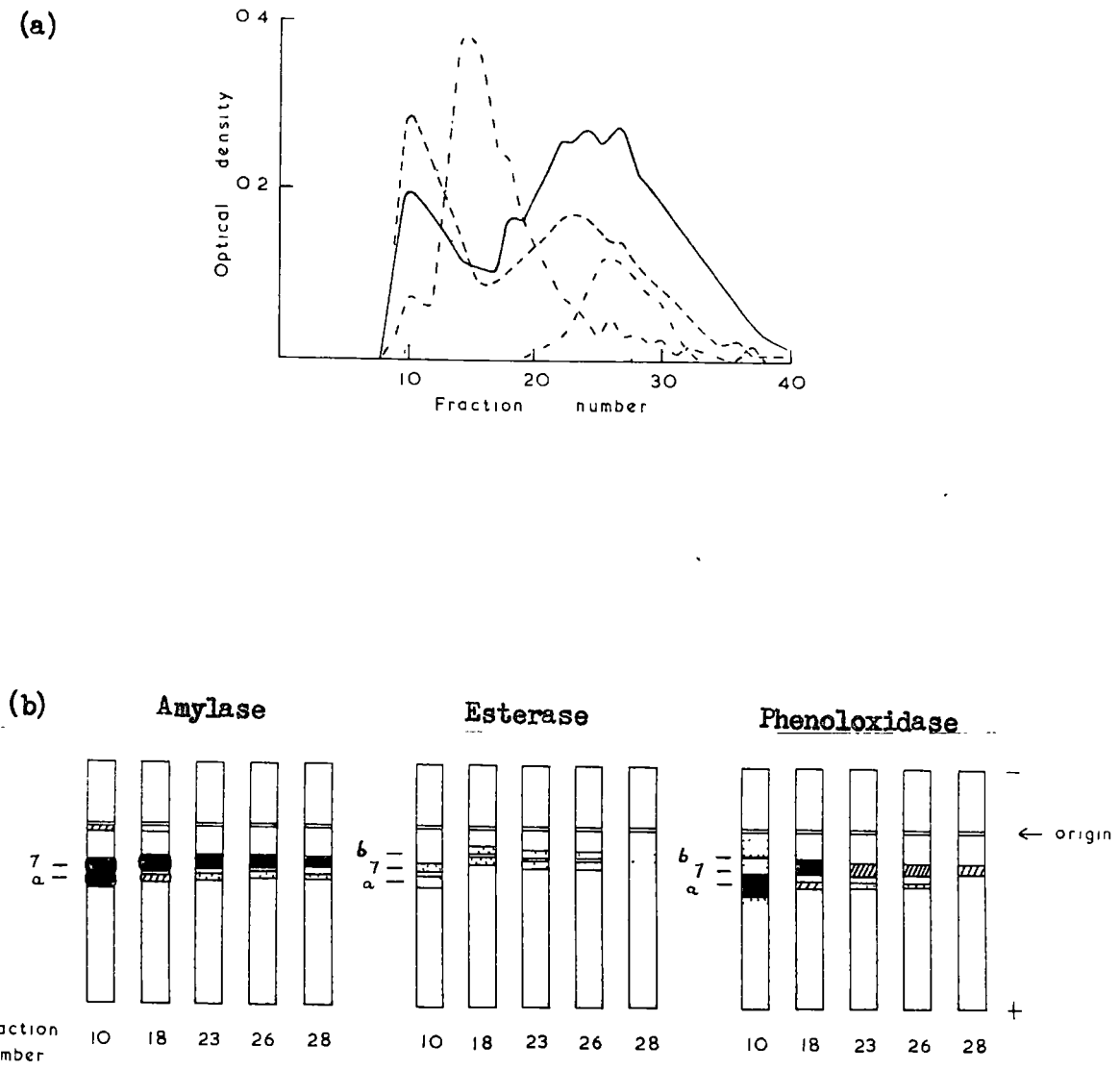


Fig. 56. (a) Elution profile of trypsin treated mucus, using Biogel P60.

——— protein

- - - - carbohydrate

-·-·-·- trypsin

-·-·- amylase

····· phenoloxidase

(b) Electrophoretograms of eluant fractions, obtained from the fractionation of trypsin treated mucus at (a).

1 fraction = 1.5ml

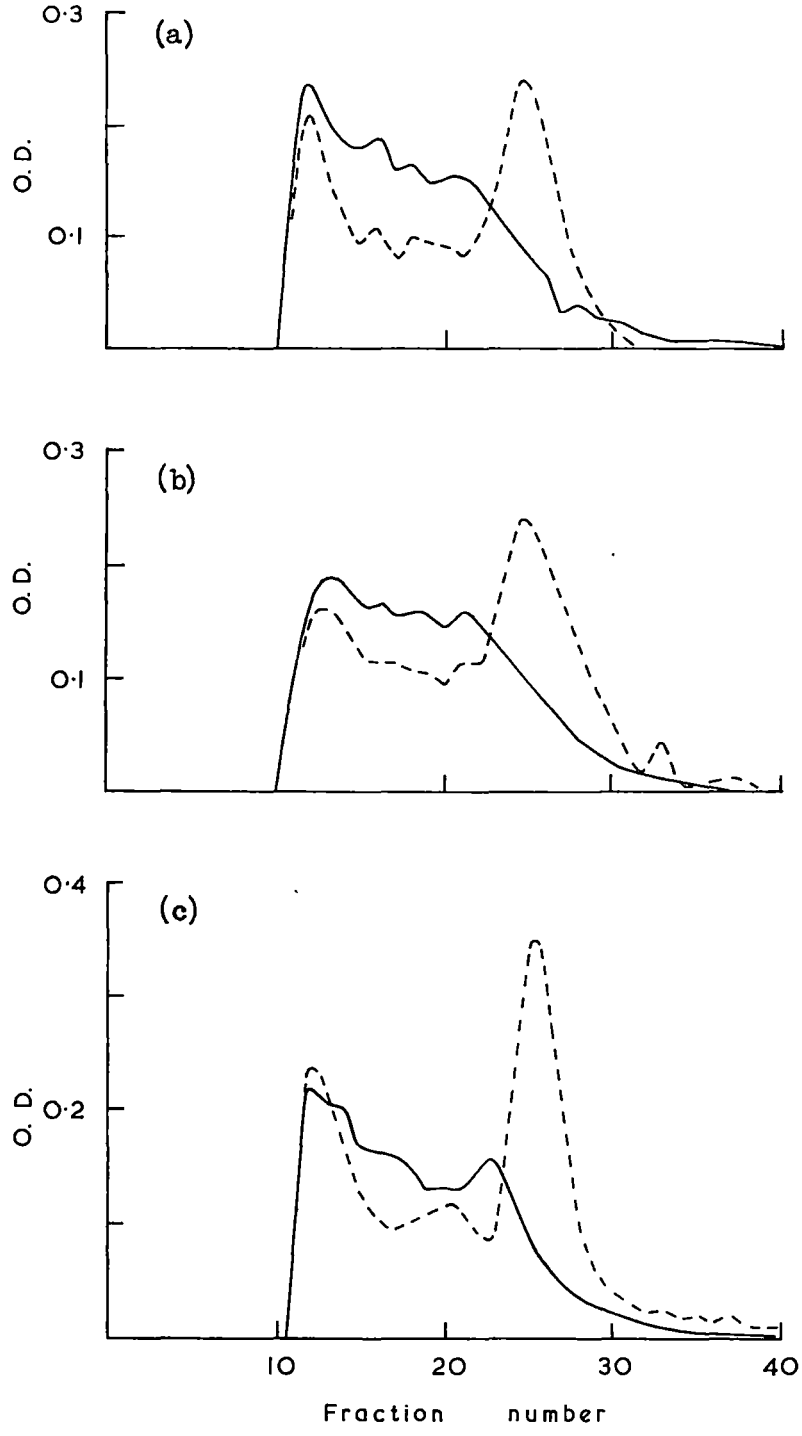


Fig. 57. Elution profiles from Biogel P6, of autoclaved mucus solutions following treatments with trypsin for (a) 24hr, (b) 36hr, and (c) 48hr. Each fraction consists of 1.5ml.

—— protein — — — carbohydrate

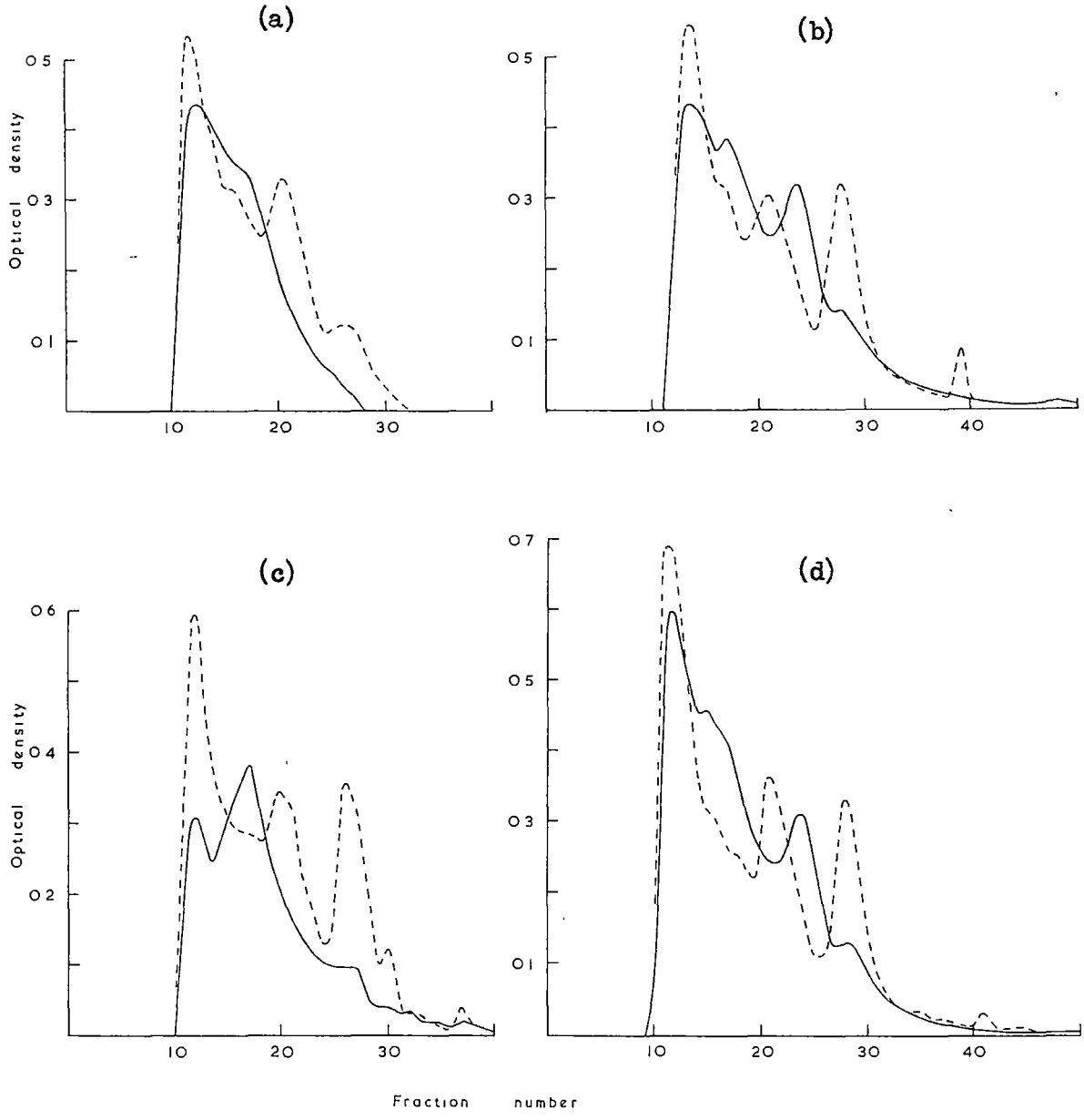


Fig. 58. Elution profiles from Biogel P6, of autoclaved mucus solutions.

(a) 24hr of treatment with papain, and elution with H_2O.

(b) no treatment, and elution with tris-HCl buffer.

(c) 48hr of treatment with papain, and elution with H_2O.

(d) 24hr of treatment with papain, and elution with tris-HCl buffer.

——— protein - - - carbohydrate

1 fraction = 1.5ml

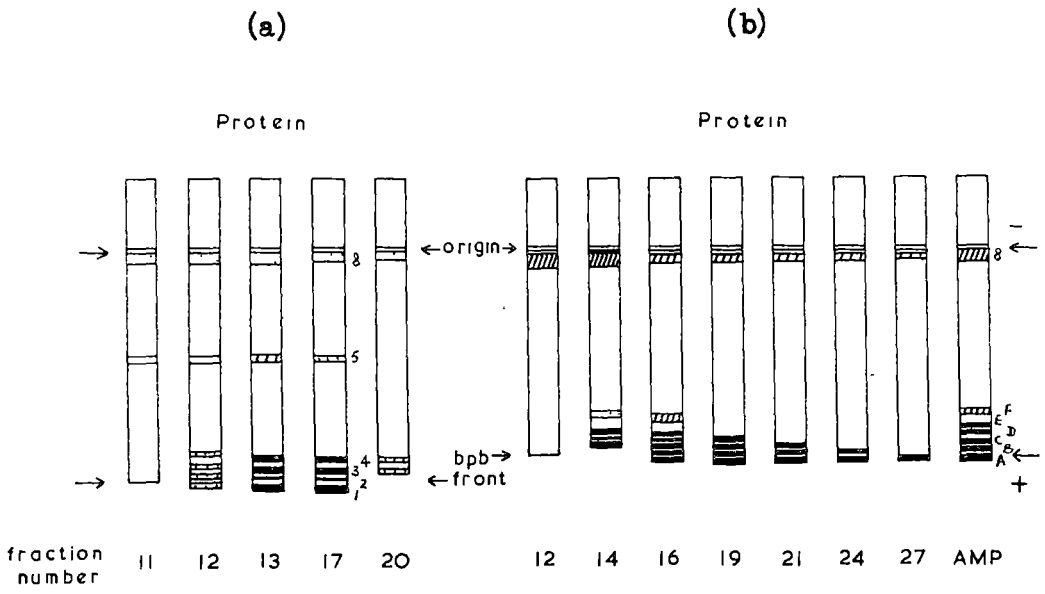


Fig. 59. Electrophoretograms of eluant fractions from Biogel P6.

(a) autoclaved mucus, from fractionation of fig. 58b.

(b) autoclaved mucus following treatment with papain for 24hr. Eluant fractions were derived from the fractionation of fig. 58d.

AMP = unfractionated, papain - treated autoclaved mucus

bpb front = bromophenol blue front

▓ /// ⁘ indicate decreasing intensity of staining

Table 20: The response of *P.radiata* twigs to solutions of autoclaved mucus following treatments with trypsin and papain.

Enzyme used	Period of treatment	AM fractions	Degree of senescence (days) 7	11	15	20
Trypsin	24hr	E	1	4	5	5
"	"	R	0	0	0	0
"	36hr	E	1	4	5	5
"	"	R	0	0	0	0
"	48hr	E	½	1	3½	4½
"	"	R	0	0	1	2
Papain	24hr	E	1½	3	4	5
"	"	R	0	½	1	1
"	48hr	E	1½	2	3½	4½
"	"	R	0	½	½	½
-	-	E	1	3	4½	5
-	-	R	0	½	1	1½
Denatured trypsin			0	0	0	0
Denatured papain			0	0	0	0
Deionised water			0	0	0	0

AM = autoclaved mucus

E = eluant fractions of autoclaved mucus, from Biogel P6

R = column residues of Biogel P6

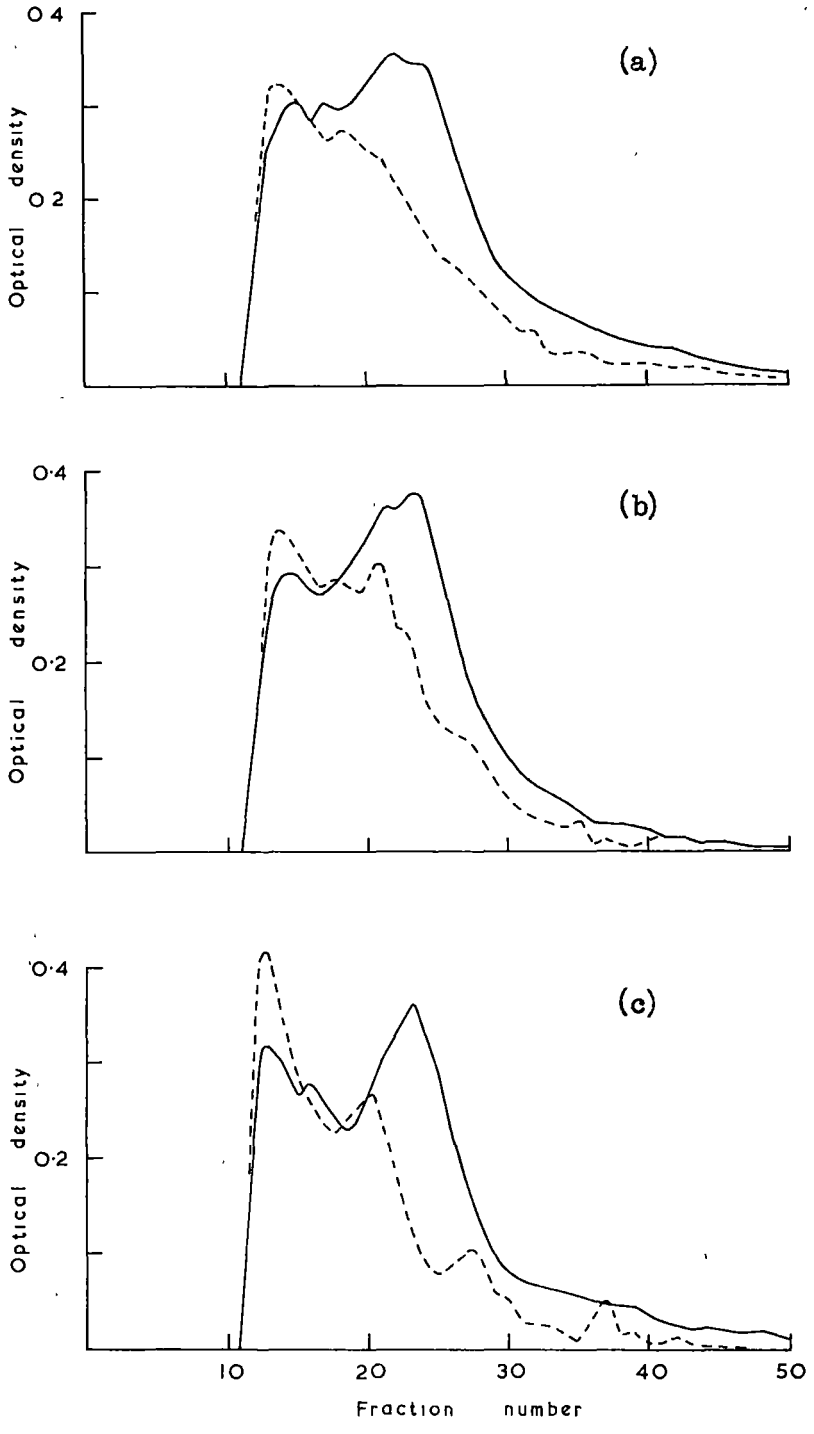


Fig. 60. Elution profiles from Biogel P6, of autoclaved mucus solutions following treatments with cellulase for (a) 24hr, (b) 36hr, and (c) 48hr. Each fraction consists of 1.5ml.

——— protein — — — carbohydrate

Table 21: The response of P.radiata twigs to solutions of autoclaved mucus following treatments with cellulase.

Enzyme used	Period of treatment	AM fractions	Degree of senescence (days)			
			7	9	11	13
Cellulase	24hr	E	2	3	4	5
"	"	R	0	1½	3	4
"	36hr	E	3	3½	4½	5
"	"	R	0	½	1	1
"	48hr	E	2½	3	4½	5
"	"	R	0	0	½	½
-	-	E	1	2	3½	4½
-	-	R	0	1	2½	3
Denatured cellulase			0	0	0	0
Deionised water			0	0	0	0

AM = autoclaved mucus

E = eluant fractions of autoclaved mucus, from Biogel P6

R = column residues of Biogel P6

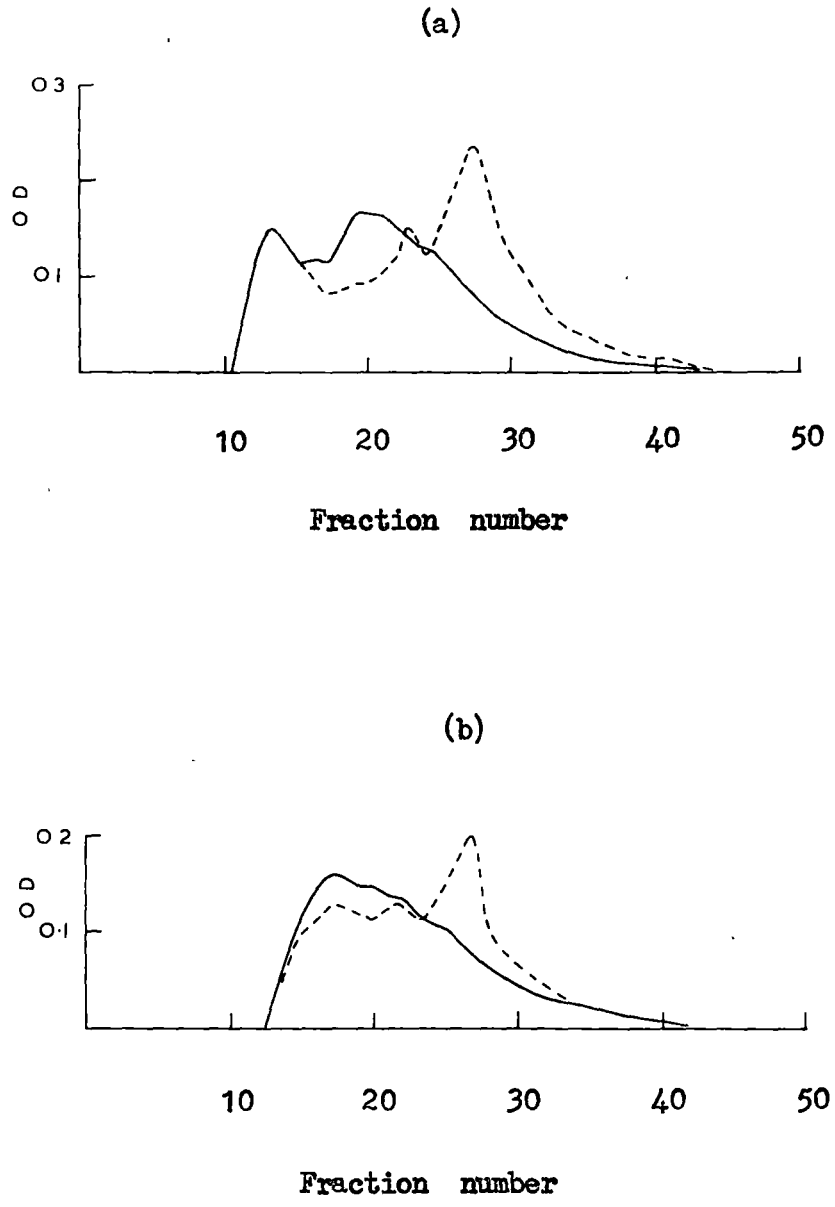


Fig. 61. Elution profiles from Biogel P10, of autoclaved mucus solutions following treatments with cellulase for (a) 24hr, and (b) 48hr. Each fraction consists of 1.5ml.

——— protein - - - carbohydrate

Ion - exchange chromatography

a. With DEAE - Sephadex, A50 (Cl^- form)

(i) using a continuous straight NaCl gradient for the elution of autoclaved mucus.

Autoclaved mucus was eluted into two major peaks of protein and carbohydrate, from DEAE - Sephadex (Figs. 62a and 64a). The protein and carbohydrate peaks of "FI" were not eluted in parallel, but appeared in the range of 0.2 - 0.45M NaCl and 0.2 - 0.55M NaCl, respectively. The second peak "FII" consisted of protein and carbohydrate which eluted in parallel, in the range of 0.55 - 0.9M NaCl. Three solutions, comprising mainly of the first protein peak (i.e., FI_a), the first carbohydrate peak (i.e., FI_b) and the second protein - carbohydrate peaks (i.e., FII), (refer to fig. 62a) were each reduced to 2ml in volume and refractionated on a small column (15ml) of Biogel P6. Their elution patterns suggest that glycopeptides of similar molecular size were present in each of the three solutions, but with FII having an additional small protein peak (fig. 62b). Electrophoresis of fractions from FI_a and FI_b revealed the presence of only one weak protein band near the origin (i.e., equivalent to band number 8 of fig. 53b), which also reacted with the P.A.S. reagent but not with any of the basic dyes. The total complement of protein bands which are normally present in whole autoclaved mucus, were located in the FII fractions (fig. 63). All of the protein bands of FII were reactive towards P.A.S., TB, AB, and AO, and in a manner similar to the results given in pages 199 to 208.

The elution of autoclaved mucus was affected by the temperature at which the fractionation was conducted, and by the gradient of NaCl present in the eluent medium. At 2°C, the separation of FI and FII was

fairly distinct, but there was a tendency for some overlapping to occur when fractionations were conducted at room temperature (compare figs. 62a and 64a). A gradual gradient of 0 - 1 M NaCl tended to produce much flattening of the FII peak but with some enhancement of the FI hexose peak, when compared with the use of a steeper gradient of 0 - 2 M NaCl (compare figs. 64a and 65).

Although the elution profiles of aged mucus solutions were more or less similar to the elution profile of autoclaved mucus, there was a trend towards the diminution of FI and especially of FII (fig. 64 b and c). This is correlated with the disappearance of small glycoprotein molecules from the aged mucus samples (refer to fig. 35a).

After being desalted on columns of Biogel P6, FI_a, FI_b and FII of fig. 62, were found to contain active physiological activities. They induced rapid desiccation of P.radiata needles, so that the dead dry twigs appeared grey - green in colour. It is suggested that the presence of residual salt in these test solutions was partly responsible for the rapid tissue desiccation. Indeed, dried samples of these test solutions were found to contain white salt crystals; the salt being derived from the eluent medium of the DEAE - Sephadex fractionation.

On repeating the bioassay of FI and FII which had been desalted by dialysis, the usual mucus syndrome was detected in the P.radiata twigs, i.e., the needles gradually turned yellow and dry, and brown. The physiological activity of FI appeared to be higher than that of FII when dilute test solutions were used, but in the presence of more concentrated solutions the relative activities of these two solutions were not readily discernible.

The effect of salt (i.e., NaCl) on P.radiata twigs was also investigated. In the presence of 20ml of 0.5N NaCl (i.e., 0.58g) the twig

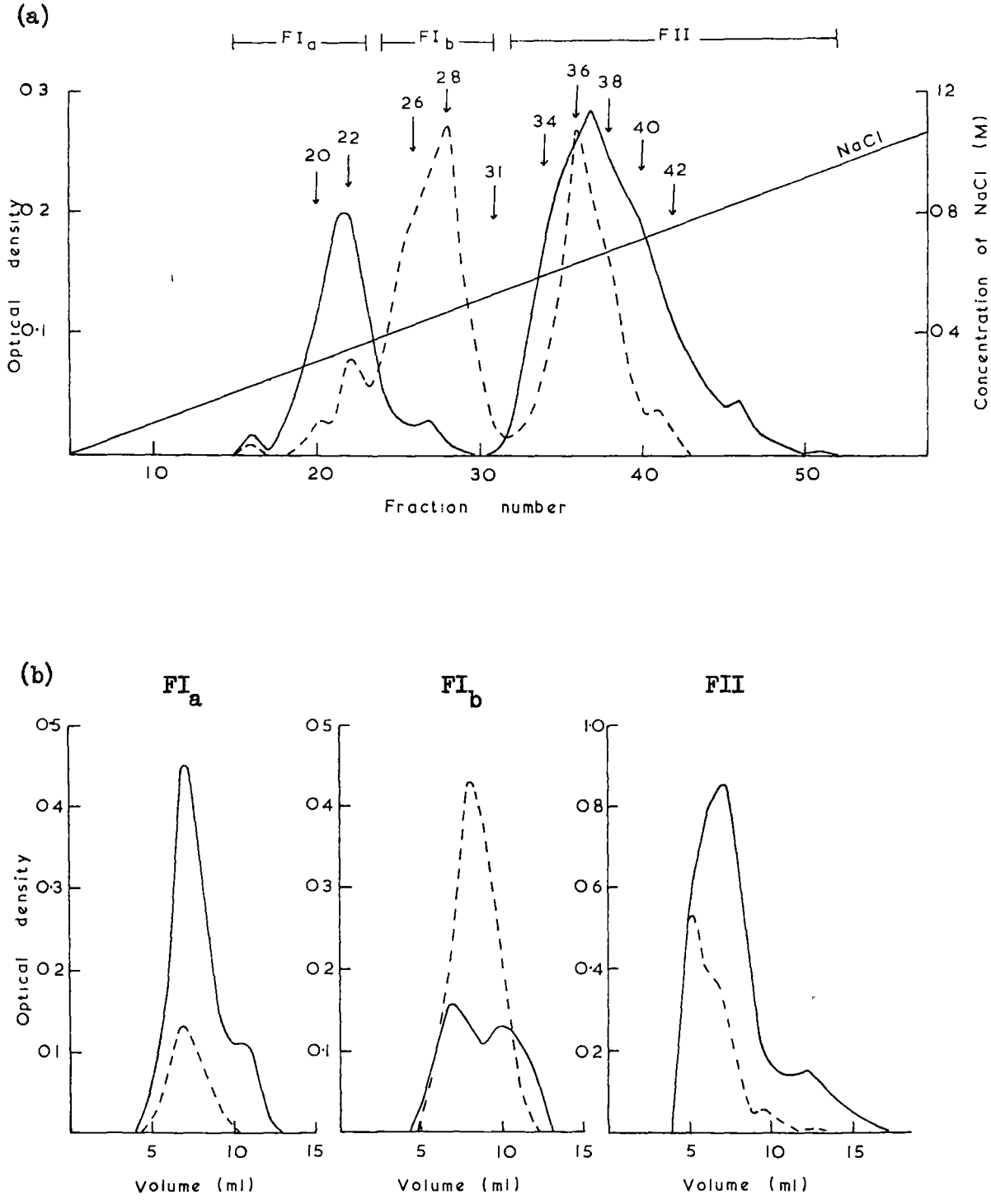


Fig. 62. (a) Elution profile of autoclaved mucus from DEAE - Sephadex at 2°C, with a straight NaCl gradient of 0 - 2 M. The arrowed numbers indicate the fractions which were used for electrophoresis. Each fraction consists of 1.5ml.

(b) Fractionation of FI_a, FI_b, and FII on Biogel P6.

—— protein - - - hexose

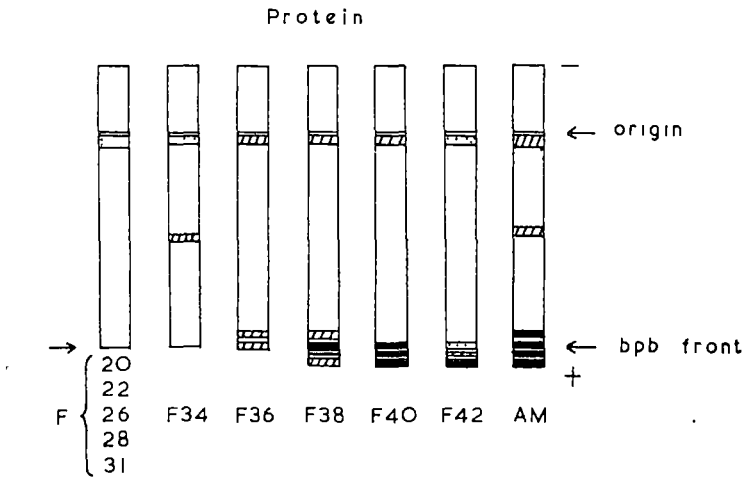


Fig. 63. Electrophoretogram of eluant fractions of autoclaved mucus, from the DEAE - Sephadex fractionation of fig. 62a.

AM = unfractionated autoclaved mucus solution

bpb front = bromophenol blue front

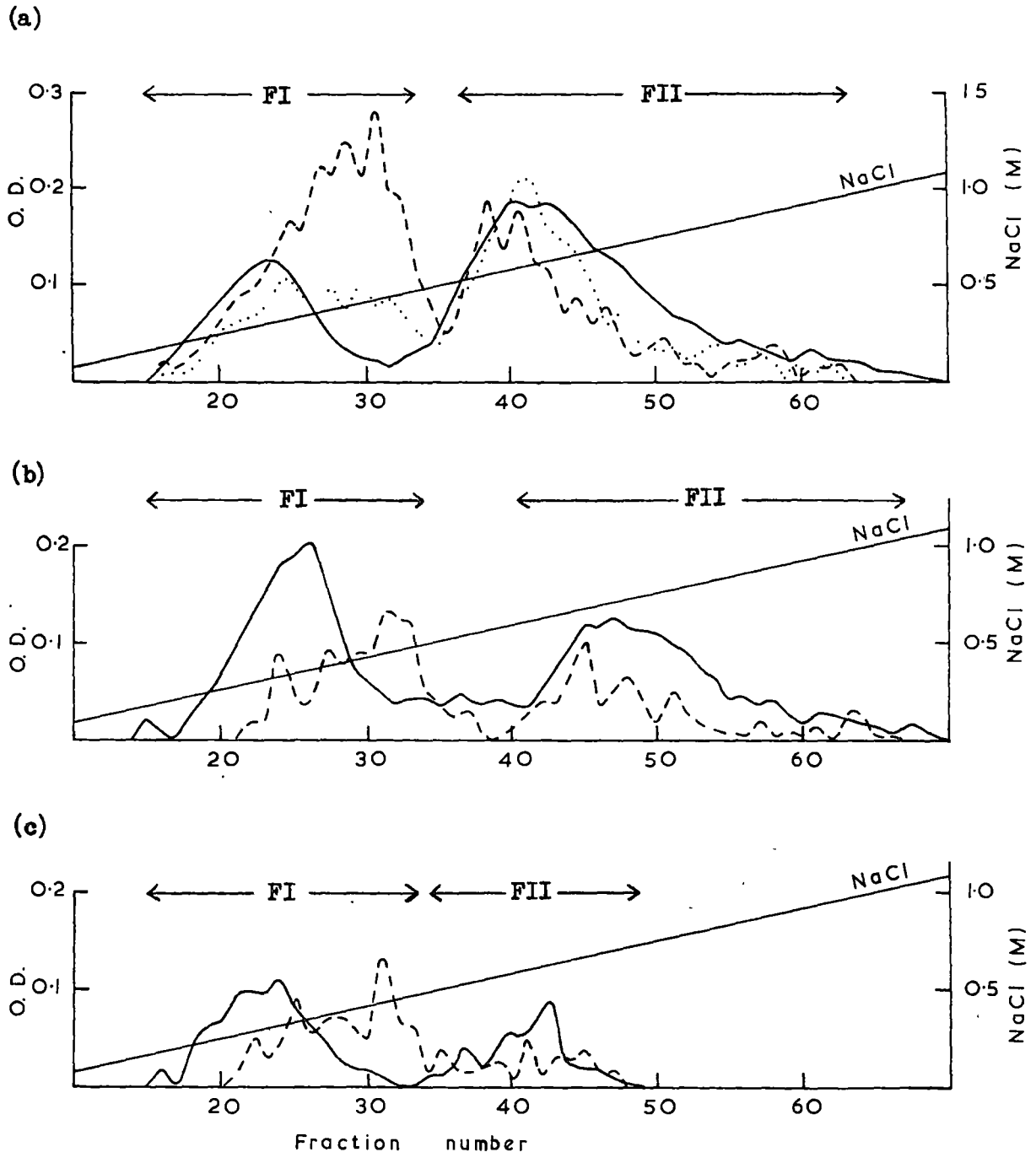


Fig. 64. Elution profiles of S.noctilio mucus solutions from DEAE - Sephadex, at room temperature (about 20°C), with a straight NaCl gradient of 0 - 2 M. Each fraction consists of 1.5ml.

(a) autoclaved mucus solution.

(b) aged mucus solution, stored at 37°C for 125hr.

(c) aged mucus solution, stored at 37°C for 250hr.

—— protein — — — hexose .... hexuronic acid

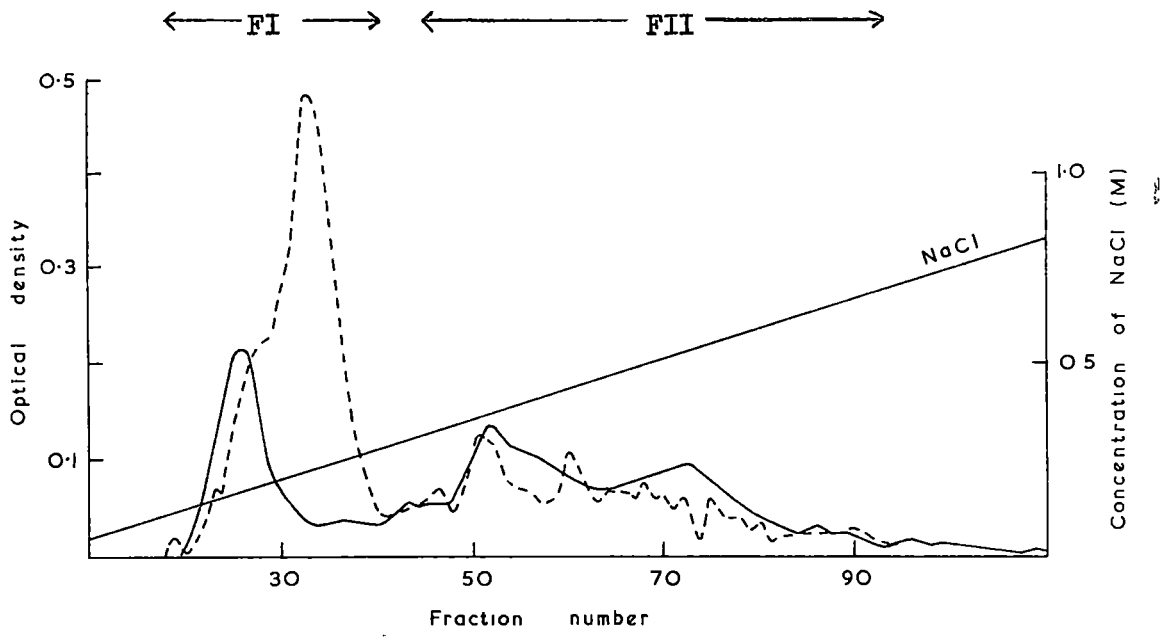


Fig. 65. Elution profile of autoclaved mucus from DEAE - Sephadex, at room temperature (about 20°C), with a straight NaCl gradient of 0 - 1 M. Each fraction consists of 1.5ml.

——— protein — — — carbohydrate (i.e., hexose)

needles rapidly turned brown and dry from the basal to the proximal regions, and the whole twig was dead in a week. When 20ml of 0.25N NaCl (i.e., 0.29g) was used, the symptoms were not as severe, but desiccation and browning of the needles were observed. The rate of response of P.radiata twigs to the various test solutions is shown in fig. 66.

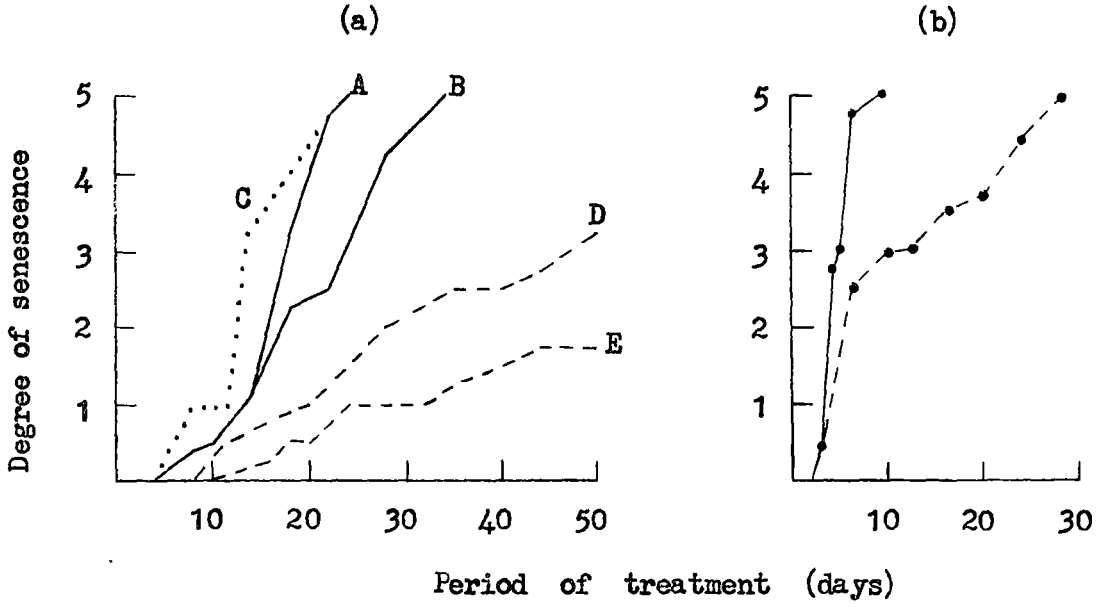


Fig. 66. (a) The response of P.radiata twigs to eluant fractions from DEAE - Sephadex, using 0.16g of autoclaved mucus for each fractionation procedure.

A = FI_a, B = FI_b, C = FII } from the fractionation of figure 62, and desalted from Biogel P6 columns.

D = FI, E = FII } from a subsequent fractionation, and desalted by means of dialysis.

(b) The effect of NaCl on P.radiata twigs.

-•- -•- 0.25M NaCl -•—•- 0.5M NaCl

The carbohydrate and aminoacid compositions of FI and FII are given in table 22. The carbohydrate fraction of FI is mainly composed of fucose and galactose with lesser amounts of glucosamine and galacturonic acid. Although the qualitative differences in aminoacids of FI and FII are small, the total concentration appears to be less in FI than in FII. This is supported by actual measurements of protein concentrations in both of these fractions; the ratio of protein in FI : FII was found to be about 1 : 2.5. The presence of hydroxyproline in FI protein suggests that it is of a collageneous type. Thus, its relatively simple sugar composition, presence of approximately equivalent amounts of acidic (i.e., glutamic acid, aspartic acid) and basic (i.e., arginine, lysine) aminoacids, its slow electrophoretic mobility, and its elution within 0.55M NaCl from DEAE - Sephadex (in the presence of a continuous straight NaCl gradient), suggest that FI is possibly a high molecular weight glycoprotein.

The polysaccharide - protein complex of FII is comparatively more complicated. Besides containing a greater variety of sugars it has a high concentration of acidic aminoacids(i.e., aspartic acid and glutamic acid) but it does not contain hydroxyproline. Therefore, it is probably not a molecular species of FI. The peak of FII eluted from DEAE - Sephadex in the region of 0.6M NaCl. Bovine vitreous hyaluronic acid eluted in the range of 0.36 - 0.43 M NaCl, with a continuous straight NaCl gradient (Berman, 1962). Together with its electrophoretic pattern, FII appears to consist of a glycoprotein and acidic polysaccharide. Results obtained from histochemical reactions of the FII complex (refer to page 259) suggest that the acidic polysaccharide is possibly non - sulphated, and is similar in some respects to vertebrate hyaluronic acid.

In earlier investigations, sulphate was detected in S.noctilio

Table 22: The carbohydrate and aminoacid compositions of FI and FII, derived from DEAE - Sephadex fractionation of autoclaved, S.noctilio mucus.

Component	Vizual estimates of relative intensities in FI	FII
fucose	2+	+
galactose	2+	5+
rhamnose	-	+
mannose	trace	+
glucose	-	+
glucosamine	+	+
galactosamine	-	+
glucuronic acid	-	+
galacturonic acid	+	+
arginine	+	+
aspartic acid	2+	4+
glutamic acid	+	2+
cysteine	-	+
glutamine	+	+
hydroxyproline	2+	-
glycine	2+	2+
alanine	+	2+
proline	2+	2+
lysine	2+	2+
serine	2+	3+
tyrosine	trace	+
valine	+	+
tryptophan	trace	+
leucine	+	2+
threonine	2+	2+
phenylalanine	+	+

mucus. An analysis of sulphate in FI and FII revealed the presence of sulphate in both of these fractions, in the ratio of 1.6 : 1, respectively. In spite of the relatively higher concentration of sulphate in FI, it had not reacted in a manner which would indicate that it was more acidic than FII, either in its reactions to histochemical stains, its electrophoretic mobility, or its elution from DEAE - Sephadex. Moreover, acid - hydrolysed solutions of FI and FII had pH values of 5 and 3.5, respectively. Thus, it appears that the glycoprotein of FI is probably sulphated; it also appears that the glycoprotein fraction of FII is sulphated, since its acidic polysaccharide was typically non - sulphated in its histochemical reactions.

(ii) using a stepwise increment in NaCl concentration for the elution of autoclaved mucus from DEAE - Sephadex.

Most of the autoclaved mucus was eluted by 0.1M NaCl, although a residual amount was eluted by 0.3M NaCl, from DEAE - Sephadex (fig. 67). There were no additional mucus fractions in the 0.5M, 1.5M, or 2.0M NaCl elutions. This elution characteristic is not typical of acid mucopolysaccharides. A mixture of authentic acid mucopolysaccharides was eluted from DEAE - Sephadex by a stepwise-increase in NaCl gradient, in the order of hyaluronic acid (at 0.5M NaCl), heparitin sulphate (at 1.25M NaCl), chondroitin sulphates (at 1.50M NaCl) and heparin (at 2M NaCl) (Schmidt, 1962).

The 0.1M NaCl eluant fractions of autoclaved mucus were found to contain the full number of glycoprotein bands which are normally present in an unfractionated autoclaved mucus sample (refer to fig. 53); an acid hydrolysate of the combined eluant fractions also contained all of the normal sugars and aminoacids (refer to table 16 and figure 44). Unfortunately, the 0.3M NaCl eluant fractions of autoclaved mucus were

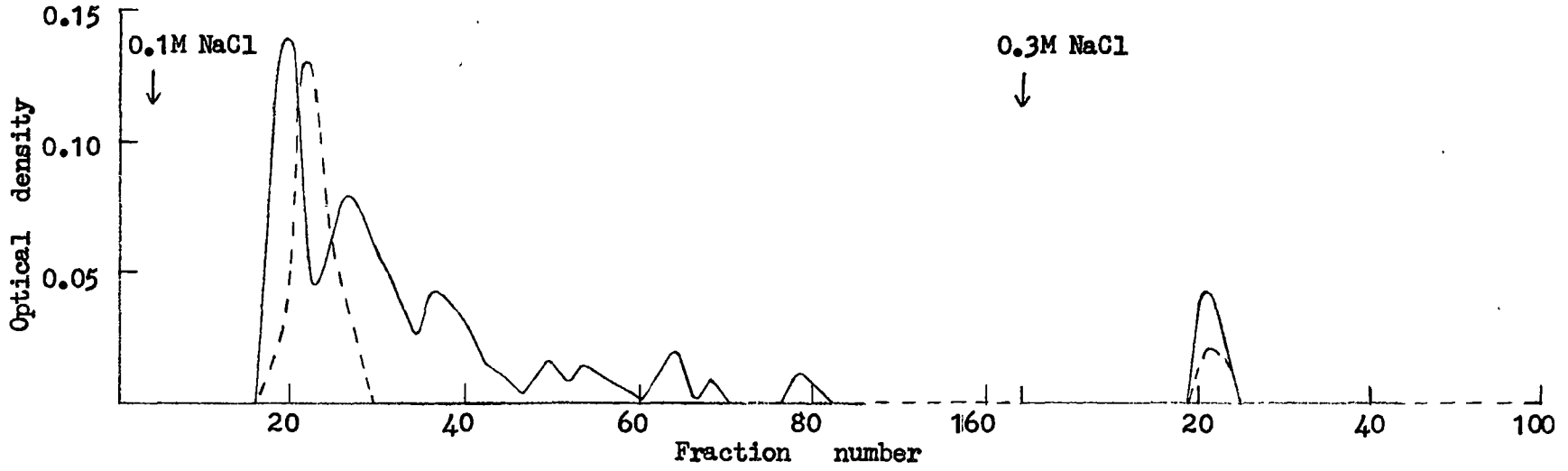


Fig. 67. Elution profile of autoclaved mucus from DEAE - Sephadex at room temperature (about 20°C), with a stepwise - increase in NaCl concentration in the eluent buffer. Each fraction consists of 2.0ml.

—— protein - - - hexose

too dilute to enable satisfactory analyses of sugars and aminoacids to be carried out.

From these analytical data, it appears that practically all the autoclaved mucus was eluted from DEAE - Sephadex in 0.1M NaCl, and that FI and FII of figure 64 had failed to separate from each other in the presence of a low ionic medium.

(iii) using a continuous straight NaCl gradient for the elution of cellulase treated autoclaved mucus, from DEAE - Sephadex.

The elution profile from DEAE - Sephadex was much altered, following treatment of autoclaved mucus with cellulase for 24hr (fig. 68). The two fractions which were arbitrarily separated, "FA" and "FB", were physiologically active (table 23).

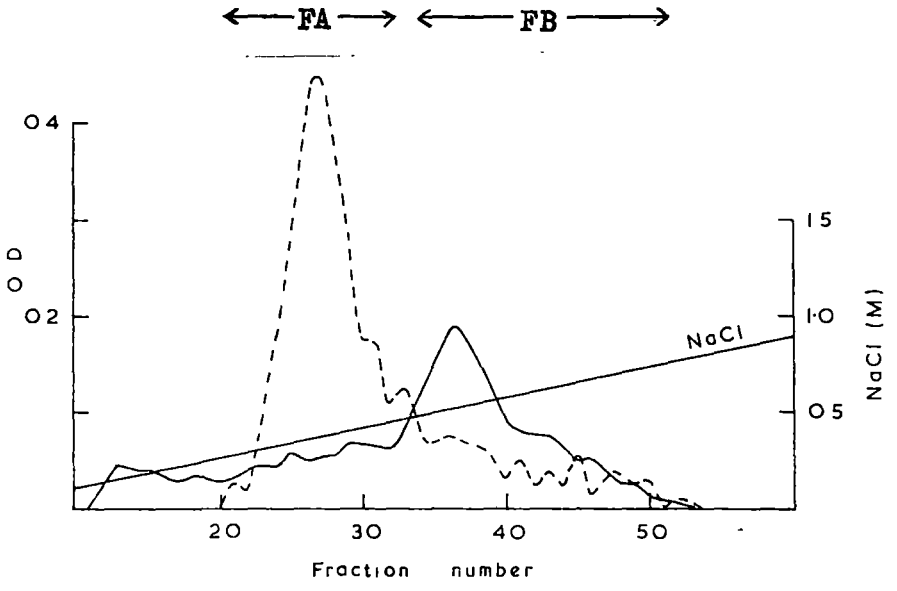


Fig. 68. Elution profile from DEAE - Sephadex at room temperature (about 20°C), with a straight NaCl gradient of 0 - 2 M, following treatment of autoclaved mucus with cellulase for 24hr. Each fraction consists of 1.5ml.

——— protein - - - hexose

Table 23: The response of P.radiata twigs to eluant fractions from DEAE - Sephadex, of autoclaved mucus following treatment with cellulase for 24hr.

	Degree of senescence after treatment			
Test solution	8 days	14 days	18 days	22 days
FA	$\frac{1}{2}$	$2\frac{1}{2}$	4	5
FB	$2\frac{1}{2}$	4	$4\frac{1}{2}$	5

b. With AG1 X2 (Cl^- form), using a continuous straight gradient of NaCl for the elution of autoclaved mucus.

Two glycoprotein complexes which differed in their compositions of sugars and aminoacids, sulphate concentration, elution characteristics, electrophoretic mobilities, and reactions to basic dyes, were obtained from the fractionation of autoclaved mucus from DEAE - Sephadex when a continuous straight NaCl gradient was used in the eluent buffer. However, the same degree of separation was not achieved when the same technique was applied to the fractionation of autoclaved mucus from a column of AG1 X2. The first fraction "FIII" was eluted in the range of 0.1 - 0.35 M NaCl, and the second fraction "FIV" was eluted in the range of 0.35 - 1.2 M NaCl (fig. 69a). Following treatments with papain (for 24hr) and CPC, autoclaved mucus was eluted into two major fractions, "FV" in the range of 0.1 - 0.3 M NaCl and "FVI" in the range of 0.5 - 0.9 M NaCl (fig. 69b). The protein and hexose fractions were eluted in parallel.

Mixtures of authentic acid mucopolysaccharides were eluted from

Dowex 1 X2 (Cl^- form) (N.B., this resin has properties which are similar to those of AG1 X2, Cl^- form) in the order of hyaluronic acid (at 0.5M NaCl), heparin monosulphate (at 1.25M NaCl), chondroitin sulphate (at 1.5M NaCl), and heparin (at 2.0M NaCl) in the presence of a stepwise - increasing NaCl concentration (Schiller, et al, 1961). Thus, the elution of autoclaved mucus from AG1 X2 (Cl^-) appears to lie within the range of NaCl concentration for hyaluronic acid. This is consistent with the weakly acidic nature of S.noctilio mucus.

Material from FIII and FV could not be detected on the electrophoretograms, as they failed to stain with amido black for protein, with the P.A.S. for carbohydrate or with basic dyes for acidic polysaccharides. However, FIV was faintly visible with all of the histochemical stains used so far, and its banding pattern was similar to that of FII of figures 62 and 63. Although FVI was not reactive with amido black, its electrophoretic pattern was also similar to that of FII of figures 62 and 63, in the presence of P.A.S. and basic dyes.

There were slight differences in the sugar composition of FIII and FV, and between these fractions and FI of figure 62 (compare tables 22 and 24). However, sugars present in FIV, FVI and FII were more or less similar although slight variations in concentrations were apparent. One of the most obvious causes for such variations is due to the different filtration properties of DEAE - Sephadex and AG1 X2.

The sulphate content of FIII and FIV occured in the ratio of 1.38 : 1.0, respectively. This is consistent with the ratio of 1.16 : 1.0 for FI : FII.

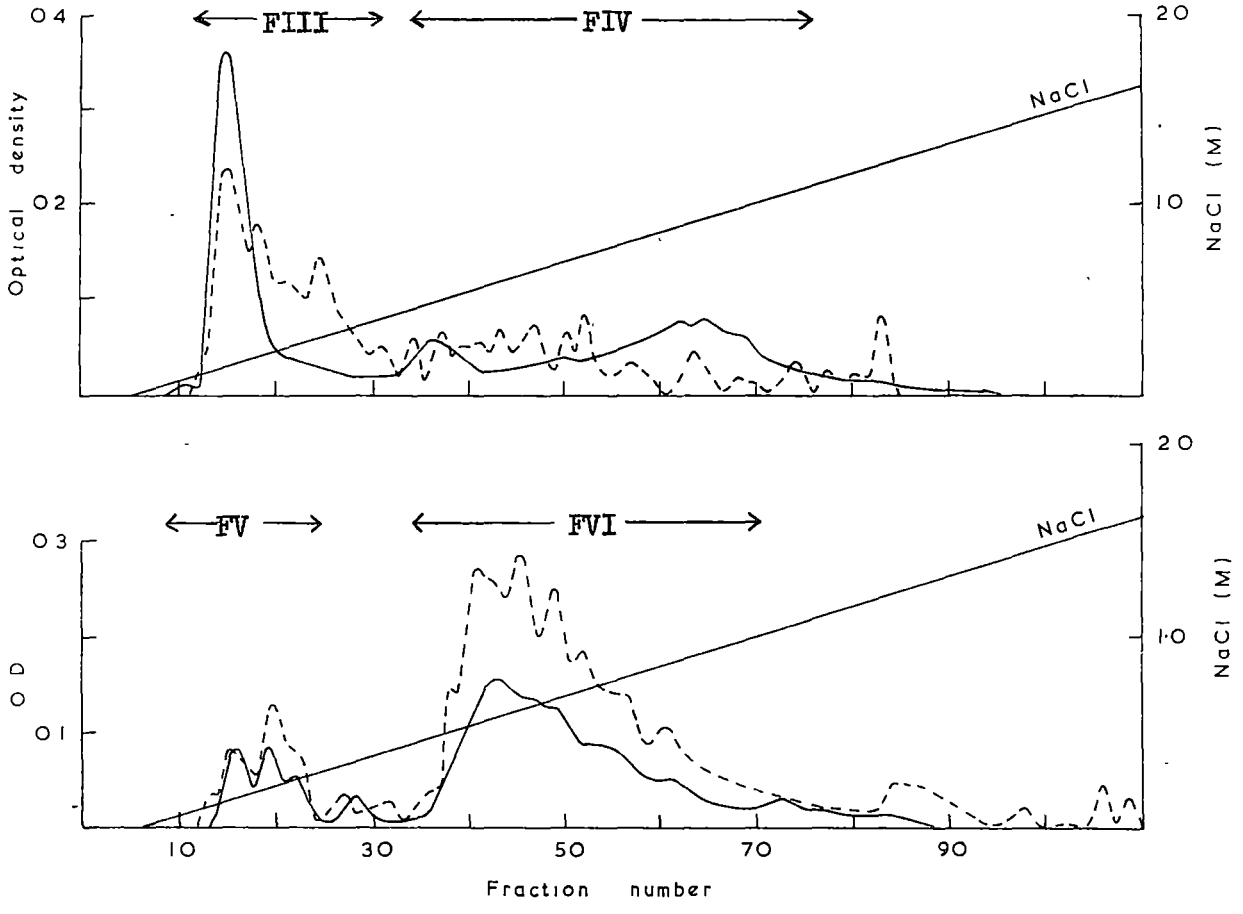


Fig. 69. Elution profiles of autoclaved mucus from AG1 X2 (Cl^- form), at room temperature (about 20°C).

(a) autoclaved mucus.

(b) autoclaved mucus, following treatment with papain for 24hr. at 60°C.

—— protein - - - hexuronic acid

1 fraction = 1.5ml

Table 24: The sugar components of eluant fractions of autoclaved mucus from AG1 X2 fractionations, as revealed by TLC. These eluant fractions were derived from the fractionations of figure 69.

Sugar	Vizual estimates of relative intensities in			
	FIII	FIV	FV	FVI
rhamnose	-	-	-	+
fucose	2+	+	+	+
mannose	-	+	+	+
glucose	+	+	±	3+
galactose	2+	3+	2+	4+
glucosamine	+	+	+	+
galactosamine	-	2+	-	2+
glucuronic acid	+	+	+	+
galacturonic acid	+	+	±	+

A brief study of the mucus secretions of two other siricids

Mucus secretions contained in glass or plastic vials in frozen form, and stored for periods of two or three years prior to use, were kindly supplied by Dr. J.P. Spradbery, formerly of the Entomology Division, C.S.I.R.O., Hobart. Mucus from two species of Xeris, three species of Urocerus, and six species of Sirex were supplied, and of these, only X.spectrum and U.gigas mucus were in sufficient quantities for use in fractionations and bioassays.

Mucus solutions of Urocerus gigas and Xeris spectrum (origin: U.S.A.) were of a light brown colour and contained substantial amounts of white, membrane - thin flecks which were probably extraneous matter. These white flecks were insoluble in cold or hot water, they did not dissolve during autoclaving, but they readily adhered onto the sides of the glassware. Unlike the mucus from S.noctilio, mucus from U.gigas and X.spectrum failed to completely dissolve in water at 2°C, with continuous stirring for four days. The water - soluble mucus was collected by filtration on sintered glass and used for fractionation on Biogel P60, whilst the insoluble mucus was treated with activated papain for 25hr (for U.gigas), or for 48hr (for X.spectrum) before being similarly fractionated on Biogel P60. Another sample of whole mucus was autoclaved and used for fractionation on Biogel P6. Finally, all of the eluant fractions were used for bioassays.

The results of Biogel fractionations of U.gigas and X.spectrum mucus solutions are given in figs. 70 and 71; their elution profiles differ from those of S.noctilio mucus solutions. In U.gigas mucus, the major protein and carbohydrate peaks were eluted more or less in parallel, but in X.spectrum mucus there appeared to be a high molecu-

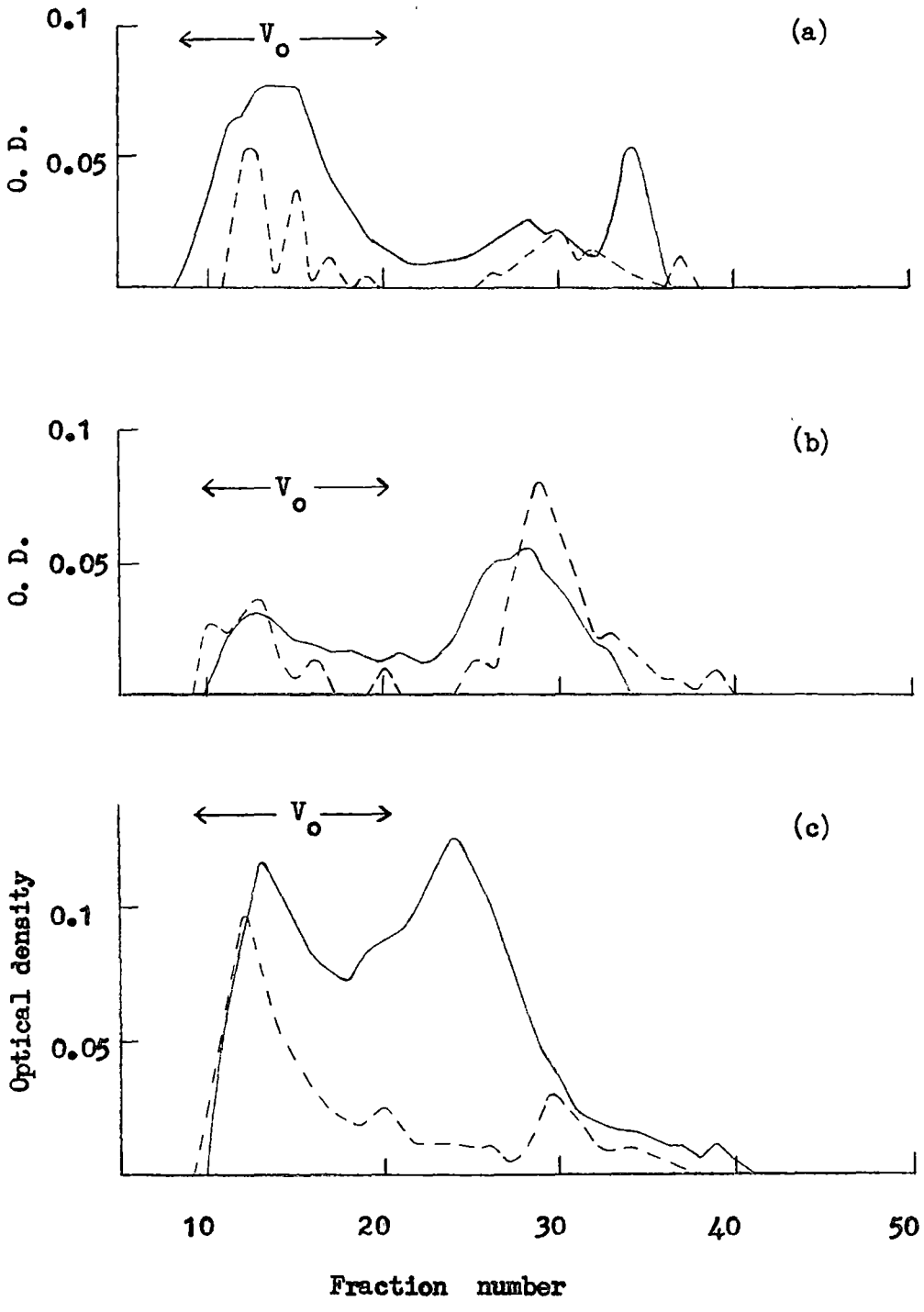


Fig. 70. Elution profiles of U.gigas mucus solutions from Biogels.

(a) Water - soluble raw mucus, from Biogel P60.

(b) Papain - treated water - insoluble mucus, from Biogel P60.

(c) Autoclaved mucus, from Biogel P6.

——— protein - - - carbohydrate

1 fraction = 1.5ml

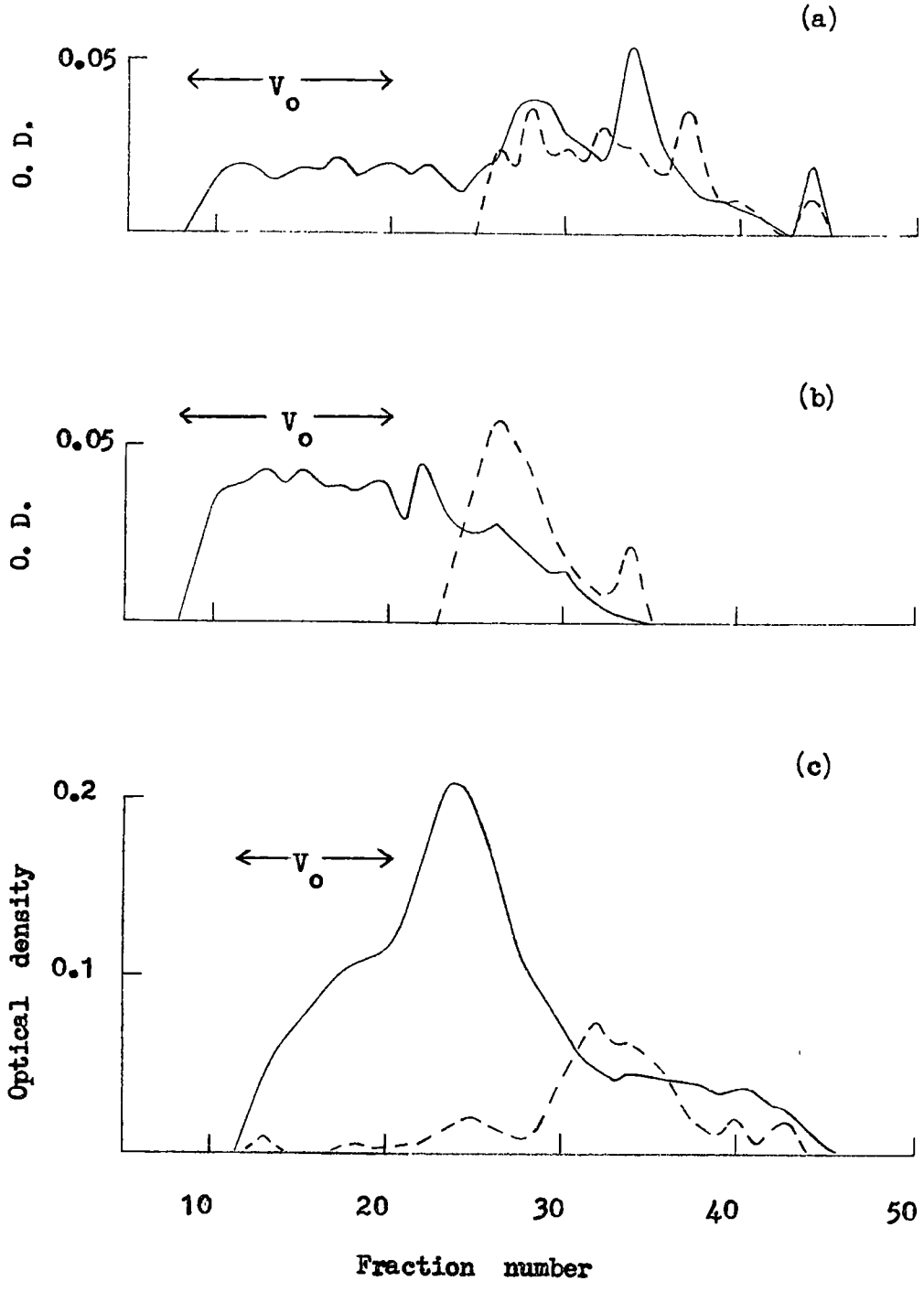


Fig. 71. Elution profiles of X.spectrum mucus solutions from Biogels.

(a) Water - soluble raw mucus, from Biogel P60.

(b) Papain - treated water - insoluble mucus, from Biogel P60.

(c) Autoclaved mucus, from Biogel P6.

——— protein - - - carbohydrate

1 fraction = 1.5ml

lar weight protein component which appeared in the void volumes of both Biogels P6 and P60, with the protein - polysaccharide component appearing in the elution volume. The mucus secretions of these two siricid species were non - phytotoxic on P.radiata (table 25), and probably indicates a high specificity of wasp to host tree species. Further studies on the mucus secretions of U.gigas and X.spectrum were not possible because of the limited supply of mucus.

Table 25: The response of P.radiata twigs to mucus secretions of S.noctilio, U.gigas, and X.spectrum.

Source of mucus	Type of mucus	Biogel column fractions	Degree of twig senescence			
			9 days	16 days	23 days	31 days
S.noctilio	Raw	P60 eluants	0	2	4	5
"	"	P60 residues	½	1	3	5
"	Autoclaved	P6 eluants	½	2	4	5
"	"	P6 residues	0	1	2	2
U.gigas	Raw	P60 eluants	0	0	0	0
"	"	P60 residues	0	0	0	0
"	Papain -	P60 eluants	½	½	½	0
"	treated	P60 residues	0	½	½	0
"	Autoclaved	P6 eluants	0	0	0	0
"	"	P6 residues	0	0	0	0
X.spectrum	Raw	P60 eluants	0	0	0	0
"	"	P60 residues	0	0	0	0
"	Papain -	P60 eluants	0	0	0	0
"	treated	P60 residues	0	0	0	0
"	Autoclaved	P6 eluants	0	0	0	0
"	"	P6 residues	0	0	0	0

Discussion

Studies based on a variety of treatments, including enzymatic digestions, gel filtration, ion - exchange chromatography, polyacrylamide gel electrophoresis, and comparisons of sugar and aminoacid compositions of the crude and partially purified mucus samples, suggest that the mucus secretion of S.noctilio is a fairly homogeneous complex of polysaccharides and protein. The molecular weight of raw mucus is estimated to lie between 60,000 - 100,000, from studies involving a series of fractionations with Biogels (P series). This is only an approximate value as other more precise methods of molecular weight estimation have not been employed.

Some aspects of these investigations have highlighted the neutral properties of the autoclaved mucus, e.g., 1) the presence of a band of very low electrophoretic mobility which only reacted with amido black (for protein) and P.A.S. (for carbohydrate), but not with any of the basic dyes. 2) the bulk of the mucus did not form an insoluble complex with cetylpyridinium chloride but instead remained in solution, in 0.04M NaCl. Acid mucopolysaccharides form insoluble complexes with cetylpyridinium chloride, which redissolve at higher concentrations of NaCl. The CPC complexes of hyaluronic acid, chondroitin sulphate and heparin, were soluble in 0.4M, 1.2M, and 2.1M NaCl, respectively (Schiller, et al, 1961). 3) it was almost totally eluted from DEAE - Sephadex in 0.1M NaCl, in a constant ionic medium. In the presence of a stepwise - increasing NaCl concentration, hyaluronic acid was eluted in 0.5M NaCl, chondroitin sulphates were eluted in 1.0M NaCl, and heparin was eluted in 2.0M NaCl (Schmidt, 1962). 4) when a continuously increasing straight NaCl gradient was used, a significant proportion

of mucus was eluted from DEAE - Sephadex in the range of 0.2 - 0.55 M NaCl. Hyaluronic acid from bovine vitreous humor eluted from DEAE - Sephadex in the range of 0.3 - 0.5 M NaCl (Berman, 1962). Although resembling hyaluronic acid in this respect, the eluant fractions of autoclaved S.noctilio mucus were mainly neutral towards the basic dyes.

The acidic properties of autoclaved mucus were accentuated in its reactions with the basic dyes, the presence of sulphate, its elution in the range of 0.55 - 0.9 M NaCl from DEAE - Sephadex with a continuously increasing straight NaCl gradient, and a minor fraction of it forming an insoluble complex with CPC which redissolved in 0.4 M NaCl. Largely owing to the different techniques which were employed for the studies of S.noctilio mucus, the acidic properties were detected by Boros (1968) who then concluded on the presence of an acid mucopolysaccharide - protein complex which contained sulphate. Gaut (1970) detected the neutral properties of S.noctilio mucus and thus concluded that it is an unsulphated neutral polysaccharide. I suggest that the acidic and neutral properties of S.noctilio mucus are due to the presence of two different groups of polysaccharide - protein complexes.

Indeed, the use of an anionic exchanger, DEAE - Sephadex, together with a continuously increasing NaCl concentration caused the separation of at least two major complexes, "FI" and "FII", from autoclaved S.noctilio mucus. Similarly, the fractionation of autoclaved S.noctilio mucus through AG1 X2 gave rise to two major polysaccharide - protein complexes, "FIII" and "FIV", or "FV" and "FVI" from a papain - treated autoclaved mucus sample.

The first of the polysaccharide - protein complexes to be eluted from DEAE - Sephadex, "FI", appeared to be a sulphated glycoprotein

which consisted mainly of fucose and galactose, with galacturonic acid and glucosamine also being present. It was located near the origin of the electrophoretogram as a single band which reacted only with amido black and P.A.S., but not with any of the basic dyes. Thus, "FI" is essentially a glycoprotein, of a collageneous type, because of the presence of hydroxyproline.

The second major polysaccharide - protein complex to be eluted from DEAE - Sephadex, "FII", was also sulphated and appeared to be made up of at least eight glycoprotein bands with molecular weights ranging from 5,000 to more than 60,000. Although sulphated, its reactions with the basic dyes were typically those of the unsulphated acid mucopolysaccharides, namely, those of hyaluronic acid. Thus, S.noctilio mucus was orthochromatic in toluidine blue at pH 3 - 7, it stained red with acridine orange in the presence of less than 0.01M NaCl, it stained blue - green with alcian blue at pH 3 - 7, and its reactions with alcian blue appeared to be mediated through its carboxyl groups.

The monosaccharide composition of the various mucopolysaccharides is given in table 26 (from Brimacombe and Webber, 1964). In view of the presence of five hexoses (i.e., rhamnose, fucose, mannose, glucose and galactose), two hexuronic acids (i.e., glucuronic acid and galacturonic acid), and two hexosamines (i.e., glucosamine and galactosamine) in "FII", it appears to be a conjugate of (sulphated) glycoprotein and a hyaluronate type of acid mucopolysaccharide. The high resistance of S.noctilio acid mucopolysaccharide to mammalian hyaluronidase is possibly due to a protective effect of the glycoproteins present in the complex.

The physiological activity of both polysaccharide - protein com-

Table 26: The monosaccharide composition of mucopolysaccharides, taken from Brimacombe and Webber, 1964.

Mucopolysaccharides	Monosaccharide components
Chitin	2-acetamido-2-deoxy-D-glucose
Hyaluronic acid	D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose
Chondroitin	D-glucuronic acid, 2-acetamido-2-deoxy-D-galactose
Chondroitin sulphate A	D-glucuronic acid, 2-acetamido-2-deoxy-4-O-sulpho-D-galactose
Chondroitin sulphate B	L-iduronic acid, 2-acetamido-2-deoxy-4-O-sulpho-D-galactose
Chondroitin sulphate C	D-glucuronic acid, 2-acetamido-2-deoxy-6-O-sulpho-D-galactose
Heparin	D-glucuronic acid, 2-deoxy-2-sulphoamino-D-glucose. (There are O-sulphate groups in both residues).
Keratosulphate	D-galactose, 2-acetamido-2-deoxy-6-O-sulpho-D-glucose
Heparitin sulphate	D-glucuronic acid, 2-deoxy-2-sulphoamino-D-glucose, 2-acetamido-2-deoxy-D-glucose
Teichuronic acid	D-glucuronic acid, 2-acetamido-2-deoxy-D-galactose
Blood group substances	L-fucose, D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose

plexes was not apparently connected with the acidic polysaccharides, since the ability of "FII" to react with basic dyes had no additive effect on its physiological activity. On the contrary, "FII" contained less physiological activity than "FI". Glycoproteins are therefore assumed to be responsible for the induction of tree senescence, and "FII" possibly contains less glycoproteins (therefore less physiological activity) than "FI". However, glycoproteins present in "FI" and "FII" may not necessarily be the same because of differences in their sugar and aminoacid compositions. These glycoproteins remained active even after they were reduced to a molecular weight of 2,000. Below this value, the physiological activity was abolished. The physiologically active glycoproteins were dialysable and susceptible to proteolytic digestions.

In the vertebrates, a sharp distinction occurs between glycoproteins and mucopolysaccharides. These glycoproteins are usually branched structures which contain acetyl hexosamines, neutral hexoses, methyl pentoses, but not uronic acids; sialic acid may occur as a prosthetic group, and ester sulphate is occasionally present. The mucopolysaccharides are usually linear structures which contain acetyl hexosamines, uronic acid residues, and ester sulphate groups. In invertebrates, the distinction between glycoproteins and mucopolysaccharides is vague, so that glycoproteins may contain uronic acids and ester sulphate, whilst mucopolysaccharides may not contain hexosamines or uronic acids (Hunt, 1970). The survey on insect polysaccharide - protein complexes given earlier in Part II of this thesis, has shown that mucoprotein, glycoprotein, neutral mucopolysaccharide and acid mucopolysaccharides either occur singly or in mixtures, in various parts of the insect body. So

far, the work of Estes and Faust (1964) on the midgut mucopolysaccharides of the greater wax moth (Galleria mellonella) has shown a strong resemblance between this insect mucopolysaccharide and mammalian hyaluronic acid, with regards to the elution pattern from ECTEOLA - cellulose, presence of N-acetyl groups, glucuronic acid and glucosamine in the approximate ratio of 1 : 1 : 1, and absence of sulphate.

The macromolecular structure of native S.noctilio mucus undergoes spontaneous disaggregation with loss of viscosity, unless it is stored at a freezing temperature. Disaggregation may also be effected with heat, or with dilute inorganic salt solutions, but not with urea. A viscose preparation isolated from the egg jelly coats of Arbacia lixula (Echinodermata) by anion - exchange chromatography on Amberlite IR 4B, was found to be a fucan sulphate which was not metachromatic in toluidine blue but contained sperm agglutination activity. In the presence of a dilute NaCl solution, this viscose fucan sulphate solution lost 70% of its viscosity, whilst a dilute solution of $CaCl_2$ caused a 60% dissociation (Monroy, et al, 1954). These viscosity properties are typical of polyelectrolytes, and the insusceptibility of fucan sulphate to urea was also consistent with its polyanionic character. The egg jelly coat substance (i.e., fertilisin) of Arbacia punctulata (with an estimated molecular weight of 300,000) and Echinarachnius parma (Echinodermata) behaved as polyanions with a high charge and high electrophoretic mobility; their sedimentation properties suggested that they were probably elongated molecules (Tyler, 1956).

As a result of disaggregation, the loss in viscosity of S.noctilio mucus solutions was accompanied by displacements in the elution profiles from Biogel columns, with release of additional chemical group-

ings (refer to table 3), and consequent increases in the protein : carbohydrate ratios (e.g., the protein : carbohydrate ratio for fresh mucus was 2 : 1, for aged mucus was 3 : 1, and for autoclaved mucus was 4 : 1), the appearance of glycopeptide units less than 10,000 in molecular weight, and improvement in the ability to induce early tree senescence. It is suggested that the mucus' own enzymes are partly responsible for the process of disaggregation since the autoclaved mucus solution (without enzymatic activity) showed no further disaggregation beyond the dissociation of the heat - labile bonds; a raw mucus solution stored at 25°C continued to lose its viscosity and became nearly as fluid as deionised water after about ten days. Although most of the mucus' enzymes (namely, amylase, phenoloxidase and proteolytic enzyme) became less active with prolonged storage, its esterase activity was improved with storage. Some of these enzymes were characterised and found to resemble β-amylase, laccase, and A-esterase in their reactions. A very active acid phosphatase activity was also detected. It was about five times as active as A-esterase. An acid phosphatase is a specific type of esterase which is actively involved in tissue degeneration. A peak in acid phosphatase activity was correlated with the maximum number of autophagic vacuoles (lysosomes) in the midgut epithelium of cockroaches (Couch and Mills, 1968). Gaut (1970) also detected A-esterase and an active acid phosphatase activity in S.noctilio mucus, but not of phenoloxidase, catalase, peroxidase, B-esterase, alkaline phosphatase and ATPase.

Besides the enzymes of the mucus, the tree's enzymes and xylem sap may also contribute towards the disaggregation of the mucus. It is envisaged that a slow process of disaggregation of mucus from the

oviposition tunnels gives rise to a continuous supply of physiologically active subunits over an extended period of time, to ensure the gradual weakening of the tree's system. This may account for the "conditioning effect" of mucus on P.radiata referred to earlier by Coutts (1969b) and Fong and Crowden (1973). The role of mucus was seen to induce the development of a more favourable environment for the establishment of the symbiotic fungus and to improve the chances of survival of the wasp's eggs and larvae.

The degree of susceptibility towards mucus is dependent on the physiological status of the tree. Weaker trees succumb to a smaller quantity of mucus than larger trees; younger needles and needles closest to the stem apices (presumably also containing a higher level of auxins) are less susceptible to mucus than are older needles and needles furthest removed from the apices. These observations suggest the influence of plant hormones in delaying or preventing the development of senescence symptoms. Experiments involving the use of kinetin on pine needle segments or radish leaf discs have shown this to be so. The susceptibility of radish leaf discs towards S.noctilio mucus serves to demonstrate the non - specificity of mucus in causing tissue chlorosis and necrosis.

Symptoms of mucus - induced senescence in P.radiata needles include chlorosis and desiccation. These result from the breakdown of chloroplasts, collapse of cellular structures which is a consequence of excessive water loss through the abnormally opened stomata, necrosis of phloem and vascular rays, and an altered mode of respiration. The ensuing starvation and desiccation provide suitable conditions for the development of the wasp's larvae and the fungus, and also serve to

weaken the tree's defences against these parasites.

The time required for a tree or twig to develop senescence symptoms, is also dependent on the rate with which the mucus solution is taken up into the plant; furthermore, intact trees show a seasonal dependence in this respect. Autoclaved mucus is less viscous and has a range of smaller molecular units than fresh mucus. It is taken up at a faster rate than fresh mucus and therefore causes an earlier senescence of the foliage.

The senescence - inducing polysaccharide - protein complex of S.noctilio mucus appears to be similar to some of the extracellular secretions of microbes which cause senescence of their host plants. A heat - labile lipomucopolysaccharide isolated from the culture fluids of Pseudomonas lachrymans (molecular weight of approximately 3.5×10^6) induced water - soaking of cucumber leaves and was also found to be toxic to mice (Keen and Williams, 1971). An extracellular toxin of Pseudomonas sp. which caused halo blight of timothy grass (Phleum pratense) was resolved into two analogues, each possessing equal specific activities, by cation - exchange chromatography (Taylor and Turbin, 1973). These analogues were found to contain taboxinine, but differed from each other by the presence of serine in one and threonine in the other. The host - specific toxin of Helminthosporium carbonum has an estimated molecular weight of slightly less than 700 (by Sephadex G-10 filtration), an empirical formula of $C_{32}H_{50}N_6O_{10}$, and is unstable. This instability results in loss of specific activity and appears to be associated with its loss of nitrogen and decreased solubility in water (Pringle, 1970). A protein - lipopolysaccharide from the culture fluids of Verticillium albo-atrum caused the wilting of cotton plants (Keen, et al,

1972). It had a molecular weight of about 3 X 10^6, and a ratio of polysaccharide : protein : lipid of 70% : 15% : 15%, respectively. The polysaccharide was composed of glucose, galactose, mannose and galacturonic acid (Keen and Long, 1972). Phytotoxic glycopeptides produced by Corynebacterium michiganense caused wilting of tomato plants, and were found to be acid labile, heat resistant and water soluble. Three fractions, each possessing biological activity, were separated with Sephadex G-200. Their molecular weights were estimated to be 200,000, 130,000 and 35,000. On acid hydrolysis, alanine, glycine, lysine, methionine, serine, threonine and an unknown aminoacid, were obtained from fractions I and III, but only alanine, glycine, lysine, serine and an unknown aminoacid were obtained from fraction II. However, galactose, glucose, mannose and two unknown monosaccharides, were detected in fractions I and II, whilst fraction III contained fucose in addition to the monosaccharides present in the other two fractions (Rai and Strobel, 1969). The glycopeptides produced by Corynebacterium insidiosum contained glucose, galactose, mannose and rhamnose in the approximate ratios of 5 : 5 : 10 : 1 : trace, respectively (Ries and Strobel, 1972); but glycopeptides produced by C.sepedonicum contained nine aminoacids and glucose (48%), mannose (13%), L-fucose (1%), and 2-keto-3-deoxy-gluconic acid. The presence of 2-keto-3-deoxy-gluconic acid was believed to cause acidity in the toxin (Strobel, 1970). These toxins induced rapid and general flaccidity of stem and leaf tissues, and studies involving the use of dyes, measurements of plasmolysis, electrolytes, ^3H_2O, and toxin binding, strongly suggested the primary destructive effect of the toxins on cellular membranes (Strobel and Hess, 1968). This hypothesis was further substantiated with electron microscope ob-

servations of membrane damage to chloroplasts, mitochondria, plasma membranes and the structural integrity of cell walls.

Thus, extracellular toxins which are essentially polysaccharide - protein complexes, cause desiccation and chlorosis to their host plants, probably by destroying cellular structures. In natural plant senescence, destruction of membrane integrity was arrested with applications of plant hormones (Shaw, et al, 1965; Shaw and Manocha, 1965; Waygood, 1965). An involvement of natural plant hormones or kinetin (by external application) in delaying or preventing the mucus effect in P.radiata foliage was also observed earlier. Although there is no electron microscope evidence at present, to show that mucus brings about the destruction of cellular structures by causing the breakdown of plasma membranes, anatomical and biochemical data presented in this thesis suggest the direct involvement of mucus in the senescence processes of P.radiata needles.

I have suggested that the glycoproteins of S.noctilio mucus are responsible for foliar senescence of P.radiata. Brief examinations of mucus from some of the other siricids, namely Urocerus gigas and Xeris spectrum have shown that although they contain carbohydrates and proteins, they were totally inactive in the physiological sense. This possibly indicates a high specificity of wasp to host tree species.

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APPENDIX I

Chemical tests for the detection of specific functional groupings

(Hawk, et al, 1947; and Dawson, et al, 1959)

Iodine test:

A drop of I_2/KI solution turns starch blue; unbranched glycogen chains turn blue but highly branched glycogen chains turn red.

Benedict's test:

Reagent: Benedict's reagent, being made up of

1. copper sulphate crystals	18g
2. sodium carbonate crystals (use half this amount for anhydrous salt)	200g
3. sodium or potassium citrate	200g
4. potassium thiocyanate	125g
5. potassium ferrocyanide, 5% solution	5ml
6. deionised water to make volume up to 1000ml.	

Procedure:

8 drops of test solution and 5ml of reagent are mixed, and boiled vigorously for 2min. A precipitate is formed, due to dextrose which may be red, yellow or green in colour, depending on the concentration of dextrose present.

Biuret test:

An equal volume of test solution and 10% NaOH is mixed, followed by a dropwise addition of 0.5% $CuSO_4$ solution. Proteoses and peptones give a purplish - violet or pinkish - violet colour.

Ninhydrin reaction:

5ml of test solution, adjusted to pH 5 to 7, is boiled with 0.5ml

of 0.1% ninhydrin solution for about 2min. Proteins, peptones, peptides, aminoacids and primary amines produce a blue colour.

Xanthoproteic reaction:

2ml of test solution on boiling with 1ml of concentrated HNO_3, turns yellow. An excess of NH_4OH or NaOH is then added, whereupon the solution changes from yellow to orange, due to the presence of phenyl groups. Thus, tyrosine and tryptophan but not phenylalanine are reactive with this reagent.

Millon's test:

Reagents:

A = 10g of mercuric sulphate ($HgSO_4$) dissolved in 100ml of 10% H_2SO_4, with heating. The total volume of the solution is made up to 200ml with 10% H_2SO_4.

B = 0.5ml of 0.25% sodium nitrite ($NaNO_2$) added to 5ml of reagent A.

Procedure:

5ml of dilute test solution and 4 drops of Millon's reagent (i.e., reagent B) is gradually boiled over a low heat. Phenolic compounds which are unsubstituted in the 3, 5 position, e.g., tyrosine, phenol and thymol, will turn red.

Folin's test:

A mixture consisting of equal volumes of test solution and Folin - Ciocalteau reagent is reacted with 3 - 10ml of a saturated solution of Na_2CO_3. Tyrosine and tryptophan turn blue.

Aldehyde reaction or Ehrlich test:

Reagent:

2.57% (w/v) of p-dimethylaminobenzaldehyde in 1 : 1 (v/v) solution of absolute EtOH and concentrated HCl.

Procedure:

1ml of test solution is mixed with 1ml of reagent. The presence of tryptophan (being composed of indole, benzene and pyrrole rings) causes the solution to turn red - violet.

Hopkins - Cole test (also known as the glyoxylic acid test):

To 1ml of test solution is added a dilute solution of glyoxylic acid, followed by careful stratification of concentrated H_2SO_4. A reddish violet ring formed at the junction of the two liquid layers indicates the presence of tryptophan. Pure tryptophan gives a negative reaction, except in the presence of trace amounts of ferric or cupric ions.

Copper sulphate test:

1ml of test solution is mixed with 1ml of 1% $CuSO_4$ solution. Sulphanilamide stains grey - brown, but other amines stain green, purplish brown or greenish yellow.

Ferric chloride test:

In the presence of sulphhydryl groups (-SH groups), dropwise additions of 1% aqueous $FeCl_3$ give an indigo - blue colour which disappears almost immediately. Addition of drops of 1% aqueous $CuSO_4$ causes the appearance of a transitory violet colour.

Basic lead acetate test:

To 1ml of test solution is added 4 drops of 10% aqueous lead acetate solution. The mixture is made alkaline with NaOH or KOH. On boiling, cystine or cysteine turns brown and lead sulphide is precipitated. This is also a test for -SH groups.

Excess alcohol for precipitation of protein and polysaccharide:

In the presence of excess 95% EtOH, material containing protein and carbohydrate will be precipitated from solution.

Anthrone test:

This is a general test for carbohydrates, and has been described in Appendix II, page 313.

APPENDIX II

Assay procedures

1. Enzymes

Acid phosphatase, with α-naphthylphosphate as substrate

Reagent:

20mg of α-naphthylphosphate dissolved in 100ml of 0.1M acetate buffer, pH 5.5, containing 5mM of $MgCl_2$.

Procedure:

0.2ml of sample was incubated with 2ml of substrate solution for 1hr at 25°C. The reaction was terminated by immersing the reaction tube in a boiling water bath for 5min, after which 0.5ml of H_2O was added to the cooled mixture and its O.D. read at 530nm in a spectrophotometer, against a blank solution consisting of 2ml of substrate solution and 0.2ml of H_2O.

Amylase assay with dinitrosalicylate (Bernfield, 1955)

Reagents:

A = 1g of starch dissolved in 100ml of 0.1M acetate buffer at pH 5.0 (for β-amylase); or 1g of starch dissolved in 100ml of 0.1M phosphate buffer at pH 6.9 (for α-amylase).

B = 1g of 3,5-dinitrosalicylic acid dissolved in 20ml of 2N NaOH and 50ml of H_2O at room temperature, followed by the addition of 30g of sodium potassium tartrate. The total volume of the reagent was made up to 100ml with deionised water, and the reagent was protected from atmospheric CO_2

by means of a tube of soda lime attached to the plug of the vessel containing the reagent.

Procedure:

a. for α-amylase in P.radiata needle extracts.

0.5ml of pine needle extract was incubated with 0.5ml of starch solution at pH 6.9 for 5min at 25°C. The reaction was stopped by addition of 1ml of dinitrosalicylate reagent, and the maltose colour was developed by boiling the mixture for 5min in a water bath. After diluting the cooled mixture with 10ml of H_2O, the O.D. of the red solution was determined at 520nm in a spectrophotometer against a blank solution consisting of needle extracts in phosphate buffer and reagent B.

b. for β-amylase in S.noctilio mucus.

0.1ml of mucus solution was incubated with 0.5ml of starch solution at pH 5 for 10min at 25°C. The reaction was stopped by the addition of 1ml of dinitrosalicylate reagent, and the maltose colour was developed by boiling for 10min in a water bath. The cooled mixture was diluted with 5ml of H_2O, and its O.D. was read at 520nm in a spectrophotometer against a blank solution consisting of mucus in acetate buffer and reagent B.

Calibration:

Amylase activity is measured in terms of the amount of maltose released from starch, as a result of the activity of the enzyme. For the purpose of constructing a calibration curve, a measured volume of maltose in the appropri-

ate buffer solution and reagent B, is used.

Calibration for ∝-amylase activity (applicable to readings for pine needle extracts).

Maltose concentration (M)	O.D. at 520nm
0.001	0.08
0.002	0.21
0.004	0.46
0.006	0.72
0.008	0.96
0.010	1.22

Esterase with ∝-naphthylacetate as substrate

Reagents:

A = 0.002g of recrystallised ∝-naphthylacetate was first dissolved in 0.5ml of acetone before the addition of 10ml of 0.1M phosphate buffer, pH 6.4.

B = 0.002g of Fast Blue BB (Gurr) dissolved in 5ml of H_2O. This solution is freshly prepared for each use.

Procedure:

0.2ml of sample was incubated with 2ml of reagent A for 30min at 25°C, after which 0.5ml of reagent B was added. After a further period of 1hr at 25°C, the reaction tube was immersed in a boiling water bath for 5min. The O.D. of the cooled mix-

ture was read at 440nm in a spectrophotometer against a blank solution consisting of reagents A and B and 0.2ml of H_2O.

Peroxidase assay, by a modified method of Maehly and Chance (1955).

Reagents: (these are all freshly prepared and maintained at 25°C)

A = 0.0307g of o-dianisidine dissolved in 5ml of 95% EtOH over a bunsen flame, and diluted with 95ml of 0.1M acetate buffer at pH 4.5.

B = 0.1ml of 30% H_2O_2 diluted to 10ml with deionised water.

Procedure:

Into a silica cuvette was added 1.0ml of o-dianisidine solution and 1.0ml of H_2O, followed by 0.1ml of sample solution. After the contents had been stirred, the cuvette was transferred to the spectrophotometer which had been thermostatically maintained at 25°C. The "zero reading" on both the spectrophotometer and the chart recorder to which it was attached, was set, and 0.1ml of H_2O_2 was then added to the mixture in the cuvette. After a brief, brisk stirring, the rate of enzymatic reaction (i.e., change in O.D. with time) was automatically recorded by the chart recorder, at 430nm. The blank solution consisted of enzyme and reagent A, but reagent B was substituted with an equivalent amount of H_2O.

The peroxidase activity is expressed in terms of the rate of change of O.D. per minute, for enzymes extracted from 1g of (dry) tissues.

Phenoloxidase assay with o-dianisidine as substrate

Reagent: (freshly prepared and maintained at 25°C)

0.0307g of o-dianisidine was first dissolved in 5ml of 95% EtOH over a gentle bunsen flame, and diluted with 95ml of 0.1M acetate buffer, pH 4.5.

Procedure:

To 1.0ml of substrate and 1.0ml of H_2O in a silica cuvette, was added 0.05ml or 0.1ml of sample. The rate of reaction (i.e., change in O.D. with time) at 430nm, was recorded by the chart recorder which was attached to the spectrophotometer. The blank solution consisted of 1.0ml each of substrate and H_2O, with an additional amount of H_2O to substitute for the enzyme.

The phenoloxidase activity is expressed in terms of the rate of change of O.D. per minute, per gramme of dry mucus.

Proteolytic enzyme with Azocoll as substrate

Reagent:

Azocoll, 50 - 100 mesh (Calbiochem). This is a general proteolytic substrate which has incorporated within it, a pink dye.

Procedure:

This assay depends on the release of the pink coloured dye as a result of enzymatic digestion of the substrate.

To 0.1g of Azocoll in 3ml of phosphate buffer (0.01M, pH 7.4)

in a centrifuge tube, was added 0.5ml of sample. The mixture was thoroughly stirred before being incubated at 37°C for 3hr. During this period of incubation, the tube was briefly removed from the incubator at half hourly intervals and the contents given a thorough mixing. The insoluble substrate not digested, was centrifuged down, and the O.D. of the clear supernatant was read at 520nm in a spectrophotometer against a blank solution consisting of substate suspension and H_2O, without the presence of enzyme solution, which had been given a similar treatment to the test solution.

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2. Other assay procedures

<u>α-aminoacid assay (Moore and Stein, 1954)</u>

Reagent:

2g of ninhydrin and 0.3g of hydrindantin, dissolved in 75ml of H_2O and 25ml of 4N acetate buffer, pH 5.5.

Procedure:

An aliquot of mucus solution (0.05ml, 0.1ml, or 0.2ml) was mixed with 1.0ml of reagent, and the mixture boiled in a water bath for 15min. On cooling, 5ml of 50% absolute EtOH were added and the mixture was used for O.D. determination at 570nm. The blank solution consisted of reagent and H_2O.

Calibration:

The calibration curve was constructed with L-leucine.

In the course of this work, it became apparent that hydrindantin was insoluble in 80% EtOH, acetone or H_2O, either at room temperature or when heated. As a result, an insoluble suspension of hydrindantin in ninhydrin solution was used. The colour yield of mucus or leucine in the presence of ninhydrin and hydrindantin was more intense than when ninhydrin alone was used, but a reliable calibration curve could not be obtained when these two chemicals were used together in the reactions. It was then decided to exclude hydrindantin from the ninhydrin solution, in the assay of aminoacids.

Calibration for aminoacids, using L-leucine as the reference compound and ninhydrin as the reagent

L-leucine (mg/ml)	0.05ml sample O. D.	0.1ml sample O. D.
1.8	1.6	-
1.6	1.45	-
1.4	1.39	-
1.2	1.18	-
1.0	0.92	-
0.8	0.65	1.41
0.6	0.37	1.08
0.4	0.15	0.61
0.3	-	0.37
0.2	0.001	0.14
0.1	0.0	0.007
0.08	-	0.004
0.06	-	0.001

Protein assay

a. with the Biuret reagent (Layne, 1957).

Reagent:

1.50g of $CuSO_4.5H_2O$ and 6.0g of sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) dissolved in 500ml of H_2O, were mixed with 300ml of 10% NaOH, and the mixture then diluted with H_2O to a total volume of 1000ml. The Biuret reagent was stored in a paraffin - lined bottle. (In order to prevent excessive reduction of the Biuret reagent, 0.1% of KI may be added. The presence of 0.1% KI has no detectable effect on the rate, degree or quality of the Biuret colour.)

Procedure:

1ml of sample solution was mixed with 4ml of Biuret reagent and allowed to stand for 30min at room temperature. The O.D. was read at 540nm against a blank solution consisting of H_2O and Biuret reagent.

Comments:

The Biuret method cannot be used in the presence of ammonium salts. Lipoid material in large amounts may yield a cloudy reaction mixture which can be cleared by shaking with 1½ml of diethyl or petroleum ether. The ether phase is removed by centrifugation, and the aqueous phase is used for O.D. determination.

b. with the Folin - Ciocalteau method of Lowry, et al (1951).

Reagents: (all freshly prepared for immediate use)

A = 1g of Na_2CO_3 dissolved in 50ml of 0.1N NaOH.

B = 0.03g of $CuSO_4.5H_2O$ dissolved in 6ml of 1% potassium tartrate (w/v).

C = 1ml of reagent B mixed with 50ml of reagent A.

Procedure:

0.05ml or 0.1ml of sample was mixed with 2ml of reagent C, and allowed to stand for 10min at room temperature. To this mixture was added 0.1ml of Folin - Ciocalteau reagent (B.D.H.). After a further incubation of 30min at room temperature, the intensity of the blue solution was determined as its O.D. at 500nm, against a reagent blank.

Calibration:

Bovine serum albumin, fraction IV (Calbiochem, B grade) was used as the reference compound.

Calibration of protein with bovine serum albumin

Serum albumin mg/ml	0.05ml sample O. D.	0.1ml sample O. D.
0.1	0.02	0.05
0.2	0.05	0.11
0.5	0.12	0.23
0.8	0.19	0.36
1.0	0.24	0.45
1.4	0.33	0.58
1.6	0.36	0.64
1.8	0.41	0.70
2.0	0.45	0.76
2.4	0.53	0.90
3.0	0.65	1.06
4.0	0.80	1.29

Comments:

The final colour of the product is the result of 1) the Biuret reaction of protein with copper ions in alkali, and 2) the reduction of phosphomolybdic - phosphotungstic reagent by tyrosine and tryptophan present in the treated protein. This method is applicable to most biological material, and tryptophan, tyrosine, most phenols (except nitrophenol), uric acid, guanine, and xanthine react with the Folin reagent to produce colour. However, there are two major disadvantages with this method of assay. Firstly, the amount of colour varies with different proteins, and in this respect, it is less constant than the Biuret reaction but more constant than the method of assay which depends on the ultraviolet light absorption at 280nm. The second disadvantage is due to the fact that the colour of the reaction product is not strictly proportional tothe concentration of the sample used. The advantages with this method lie in the fact that it is more convenient and is as sensitive as digestion and subsequent nesslerization; it is ten to twenty times as sensitive as the method involving ultraviolet light absorption at 280nm; it is much more specific and more accurate than the turbidity method of protein assay; and it is a hundred times as sensitive as the Biuret reaction.

Carbohydrate, with the anthrone reagent (Trevelyan and Harrison, 1952)

Reagent: (freshly prepared for each use)

0.2g of anthrone (Light & Co.) dissolved in 100ml of dilute H_2SO_4. The dilute H_2SO_4 was made by adding concentrated H_2SO_4 to deionised water in the proportions of 5 : 2, v/v, respectively.

Procedure: (applicable to S.noctilio mucus)

0.05ml or 0.1ml of sample was added to 2.5ml of anthrone reagent, and the contents heated in a boiling water bath for 10min. The cooled mixture, green in colour, was used for O.D. determination at 620nm. The blank solution consisted of the anthrone reagent and H_2O to substitute for the sample.

Calibration:

D-galactose was used as the reference compound.

Calibration with D-galactose

Galactose mg/ml	0.05ml sample O. D.	0.1ml sample O. D.
2.0	0.89	1.78
1.6	0.69	1.39
1.2	0.53	1.06
1.0	0.44	0.89
0.6	0.26	0.55
0.2	0.08	0.18
0.1	0.04	0.09
0.06	0.025	0.05
0.02	0.008	0.09
0.01	0.003	0.008
0.004	0.0	0.005

In the assay of total carbohydrates in extracts of P.radiata needles, different proportions of sample and anthrone reagent were used. D-glucose was used as the reference compound for the assay of sugars from P.radiata needles, but D-galactose was chosen as the reference compound for sugars of S.noctilio because of the presence of a significant proportion of this sugar in the Sirex mucus. The following is a procedure for the assay of total carbohydrates in P.radiata needles.

Reagents: (freshly prepared for each use)

0.2g of anthrone in 100ml of dilute H_2SO_4 (i.e., concentrated H_2SO_4 : H_2O = 5 : 2, v/v).

Procedure: (applicable to P.radiata needles)

0.1ml of sample was added to 5ml of anthrone reagent, and the contents heated for 10min in a boiling water bath. The O.D. of the cooled mixture was read at 620nm against a reagent blank.

Calibration:

D-glucose (M.W. = 180.2) was used as the reference compound.

Calibration with D-glucose

Glucose concentration (M)	O.D. at 620nm
0.001	0.15
0.002	0.32
0.004	0.67
0.006	0.98
0.008	1.27
0.010	1.57

Comments:

The active form of the reagent, anthranol (an enol tautomer of anthrone) condenses with the carbohydrate furfural derivative to give a green colour (in dilute solution) or a blue colour (in concentrated solution). The intensity of colour produced by glucose is approximately equal to the colour produced by fructose. Galactose gives 54% of the colour of glucose, and pentoses are appreciably less sensitive. Although the anthrone method is suitable for free sugars and sugar glycosides, it cannot be used in the presence of phenol or NaCl as these interfere with the colour development of carbohydrates in the anthrone reagent.

Reducing sugars, with the dinitrosalicylate reagent

Reagent:

The composition of the dinitrosalicylate reagent was exactly the same as reagent B, for amylase assay.

Procedure:

An aliquot of sample (0.1ml, 0.2ml, or 0.5ml, depending on its concentration) was mixed with 1ml of dinitrosalicylate reagent, and the mixture was boiled for 10min in a water bath. After cooling, the mixture was diluted with 1ml of H_2O, and its O.D. then determined at 520nm in a spectrophotometer, against a reagent blank.

Hexose, with phenol - sulphuric acid (Dubois, et al, 1956)

Reagents:

A = 80g of crystalline phenol dissolved in 20g of H_2O.

B = concentrated H_2SO_4 (A.R. Grade).

Procedure:

0.1ml of sample was mixed with 0.05ml of reagent A, OR 0.05ml of sample was mixed with 0.02ml of reagent A. After 3min, 2.5ml of reagent B was added, followed by careful mixing. The mixture was further incubated for 30min at room temperature. The O.D. of the orange coloured product was read at 490nm against a reagent blank, in the spectrophotometer.

Calibration:

D-galactose was used as the reference compound.

Calibration with D-galactose

0.05ml sample		0.1ml sample	
Galactose (mg/ml)	O. D.	Galactose (mg/ml)	O. D.
3.0	0.70	2.0	1.29
2.8	0.67	1.8	1.15
2.6	1.30	1.6	1.16
2.4	0.95	1.4	1.04
2.2	0.53	1.2	1.08
2.0	0.40	1.0	0.93
1.8	0.29	0.8	0.51
1.6	0.37	0.6	0.29
1.4	0.26	0.4	0.30
1.2	0.22	0.2	0.13
1.0	0.16	0.1	0.05

Comment: The presence of NaCl does not interfere with this assay procedure.

Hexosamine assay (Boas, 1953)

Reagents: The Elson - Morgan reagent, being made up of

A = 2% solution of acetylacetone (v/v) in 1N Na_2CO_3.

B = 2.57% (w/v) of p-dimethylaminobenzaldehyde in 1 : 1 (v/v) of absolute EtOH and concentrated HCl.

Procedure:

As this procedure was mainly applied to the acid eluant fractions from the Dowex 50W(H^+) column, it was first necessary to neutralise the solutions before carrying out the assay procedure. For this purpose, an aliquot of eluant fraction was titrated against 4N NaOH using 1 drop of 0.5% phenolphthalein indicator. This solution was then back - titrated against 0.5N HCl until the colour of the indicator (red) had just disappeared. The number of drops of 4N NaOH and of 0.5N HCl used, were noted, and an equivalent volume of 4N NaCl and H_2O, respectively, was added to a volume of water (equivalent to the volume of eluant fraction) which was to be used as the "blank solution" of that particular eluant fraction.

The actual assay procedure is as follows:

To 1ml of sample (or 1ml of "blank solution") was added 1ml of reagent A. The mixture was incubated at 90°C for 60min. After cooling, 2ml of absolute EtOH was added and followed by the addition of 1ml of reagent B. The mixture was then left for 2hr at room temperature, before being read against its appropriate reagent blank at 530nm, in a spectrophotometer.

Hexuronic acid, with the modified carbazole method of Bitter and Muir (1962)

Reagents:

A = 0.025M sodium tetraborate in concentrated H_2SO_4.

B = 0.125% carbazole in absolute EtOH.

Procedure:

0.2ml of sample was added to 2.5ml of reagent A, and the mixture was boiled for 10min in a water bath. After cooling, 0.2ml of reagent B was added. The mixture was boiled again for 15min, during which time a red colour developed. The O.D. of the solution was determined at 530nm against a reagent blank, in a spectrophotometer.

Calibration:

∝-D-galacturonic acid (M.W. = 194.1) was used as the reference compound.

Calibration with galacturonic acid

Galacturonic acid (mg/ml)	O. D.
0.4	1.85
0.2	0.86
0.1	0.43
0.08	0.35
0.06	0.26
0.04	0.17
0.02	0.08
0.01	0.04
0.005	0.01

Assay for sialic acid

Various methods for the detection and assay of sialic acids have been devised, and the merits of only some of these procedures are briefly outlined below.

1. Resorcinol - hydrochloric acid method of Svennerholm (1957): the influence of hexoses is eliminated by heating the reaction mixture for shorter periods of time, but, as pentoses, glucuronic acid and 2-deoxyhexoses produce strong colours with this reagent, there is a restriction in the extent of use of this particular assay method.
2. Orcinol - hydrochloric acid (Bial's reagent) method, as described by Werner and Odin (1952): ketohexoses are not distinguished from sialic acids because they both produce similar colour reactions with the Bial's reagent.
3. Direct Ehrlich reaction, as described by Werner and Odin (1952): this procedure is believed to give a true measure of sialic acids because of non - interference from hexoses, methylpentoses, uronic acids and hexosamines. However, the Ehrlich reaction is also a pyrrole reaction, and therefore cannot be used for estimating sialic acids in body fluids and tissues which contain preformed pyrroles (Svennerholm, 1957). The violet coloured reaction product has absorption maxima at 530nm and 650nm.
4. Thiobarbituric acid method of Warren (1959): unbound sialic acids may be measured by this procedure, and the assay can be used directly on acid hydrolysates without first having to neutralise their acidity. This method is claimed to be considerably more specific than the other methods described so far. The red coloured reaction product has a maximum absorption at 549nm, and interference from deoxyribose at 532nm can be accounted for, in the final calculation.

In view of the significant amount of different types of sugars in S.noctilio mucus, only two methods were employed for the detection of sialic acids in the mucus.

Determination of sialic acid with the direct Ehrlich method (Werner and Odin, 1952)

Reagents:

Recrystallised p-dimethylaminobenzaldehyde, 1g, was dissolved in 10ml of concentrated HCl and 10ml of deionised H_2O.

Procedure:

To 1ml of sample was added 1ml of reagent. The mixture was heated in a boiling water bath for 20min, and then cooled. After dilution with 5ml of H_2O, the O.D. of the solution was read at 565nm, against a reagent blank.

Determination of sialic acid with the thiobarbiturate reagent (Warren, 1959)

Reagents:

A = sodium metaperiodate (0.2M) dissolved in 9M phosphoric acid (i.e., 207ml of conc. H_3PO_4 in 1 litre solution).

B = 10% sodium arsenite dissolved in 0.1N H_2SO_4 solution containing 0.5M Na_2SO_4.

C = 0.6% of thiobarbituric acid dissolved 0.5M Na_2SO_4.

D = Cyclohexanone.

Procedure:

0.2ml of sample was mixed with 0.1ml of reagent A, followed by the addition of 1ml of reagent B 20min later. The mixture

was shaken vigorously, and after 2min, it was shaken again. 3ml of reagent C was then added and the mixture was boiled for 15min in a water bath. On cooling, the coloured solution was extracted with vigorous shaking in 4.3ml of cyclohexanone. The two liquid phases were separated by centrifugation, and the clear cyclohexanone phase was used for O.D. determination at 532nm and 549nm, against a reagent blank. The product should be red in colour.

Calculation of sialic acid concentration:

$$\text{Concentration of sialic acid } (\mu \text{ moles}) = (0.084 \times \text{O.D.}_{549nm}) - (0.031 \times \text{O.D.}_{532nm})$$

Sulphate determination

a. turbidimetric method of Dodgson and Price (1962), with modifications.

Reagents:

A = 0.1g of commercial "Davis" gelatin dissolved in 20ml of hot H_2O. This gelatin solution was allowed to stand at 4^oC for at least 6hr or overnight, before being used.

B = $BaCl_2$ - gelatin solution consisting of 0.1g of $BaCl_2$ dissolved in 20ml of reagent A. The mixture was allowed to stand for 2 - 3hr at 4^oC, before being used.

C = 3% solution of trichloracetic acid (w/v).

Procedure:

1. Test solution consisting of 1ml of mucus hydrolysate in 1ml of reagent C and 1ml of reagent B.

2. Control solution consisting of 1ml of mucus hydrolysate in 1ml of reagent C and 1ml of reagent A.

3. Reagent blanks:

   a. 1ml of dilute HCl (normality equivalent to the normality of HCl used for acid hydrolysis of mucus) in 1ml of reagent C and 1ml of reagent B.

   b. 1ml of dilute HCl (normality equivalent to the normality of HCl used for acid hydrolysis of mucus) in 1ml of reagent C and 1ml of reagent A.

Mixtures in each of the four solutions were allowed to stand at room temperature for 20min before their O.D.was read against solution (3b), at 360nm in a spectrophotometer.

$\therefore$ O. D. due to mucus sulphate = X - Y - Z, where

X = O.D. of the test solution

Y = O.D. of the control solution

Z = O.D. of the reagent blank (3a).

Calibration:

H_2SO_4 or K_2SO_4 were used as the reference compounds.

Calibration with H_2SO_4

Normality of H_2SO_4	O. D.
0.001	0.055
0.002	0.062
0.005	0.092
0.006	0.113
0.008	0.120
0.010	0.239

b. Spectrophotometric method using barium chromate (Iwasaki, et al, 1957)

Reagents:

A = Barium chromate.

This was made by combining a solution of $BaCl_2.2H_2O$ (10g/100ml) with a solution of hot potassium chromate (8g/800ml). The resulting barium chromate precipitate was washed three times with 500ml aliquots of hot deionised H_2O, and re-precipitated with 100ml of 2M NH_4OH. The washed precipitate was dried in an oven for 2hr, at 100°C, and then pulverised.

B = $BaCrO_4$ suspension.

2.5g of $BaCrO_4$ powder was added to a 200ml solution of 0.5M HAc and 0.01M HCl. The suspension was prepared the day before it was used, and stored in the refrigerator.

C = 1.85g of $CaCl_2.2H_2O$ dissolved in 500ml of 6M NH_4OH. This reagent was protected from atmospheric CO_2 with soda lime.

D = Absolute EtOH.

Procedure:

To 1ml of sample was added iml of reagent B, followed by 0.5ml of reagent C, three minutes later. 0.5ml of reagent D was then added. The mixture was stood at room temperature for 10min, and centrifuged for another 10min in a bench centrifuge. The yellow coloured supernatant was read at 370nm, against a reagent blank, in a spectrophotometer.

Calibration:

A calibration curve was constructed each time, using K_2SO_4.

Calibration with K_2SO_4

% K_2SO_4 (w/v)	O. D.
0.001	0.14
0.002	0.35
0.004	0.80
0.006	0.99
0.008	1.07
0.010	1.08

APPENDIX III

Some histochemical stains for plant tissues

Some of these stains had been mentioned elsewhere in the thesis, but others not mentioned, had been used in preliminary studies of P.radiata needle tissues and were found to be very useful. The following is an account of the diagnostic features of the stains.

Aniline sulphate.

Reagent:

0.1g of aniline dissolved in 20ml of 0.5% H_2SO_4.

Procedure:

Fresh tissue sections are immersed in 1 drop of reagent for 5min. Sections are then rinsed with H_2O and mounted in 50% glycerine.

Results:

This is a general stain for lignin, which stains yellow or greenish - yellow.

Aniline sulphate - methylene blue.

Reagents:

A = 0.1g of aniline dissolved in 20ml of 0.5% H_2SO_4.

B = 1% aqueous solution of methylene blue, diluted by 1/50 for use.

Procedure:

Fresh tissue sections are first stained in reagent A for 5min and then rinsed in H_2O. They are counterstained in

reagent B for 5min. After rinsing in H_2O, the sections are mounted in glycerine.

Results:

This is a differential stain for temporary preparations, where lignin (e.g., xylem) stains pea - green, sclereids (e.g., sclerenchyma) stains deep blue, pectin (e.g., collenchyma) stains purplish blue, and cellulose stains light blue.

Cyanin - erythrosin.

Reagents:

A = 0.1% cyanin in 50% EtOH.

B = 1% erythrosin in 70% EtOH.

Procedure:

The sections are first stained in reagent A for 15min, rinsed with 50% EtOH, and counterstained in reagent B for 1min. They are then dehydrated with a series of EtOH solutions of concentration 50%, 95% and 100%. The sections are rinsed again in 100% EtOH before being cleared in xylol, and mounted in Canada balsam or DePex.

Results:

Lignified tissues stain blue and cellulose stains red. This is a procedure for permanent preparations.

I_2/KI.

Reagent:

2g of iodine dissolved in an aqueous solution of potassium iodide (21g KI in 50ml H_2O).

Procedure:

The fresh tissue sections are immersed in a drop of reagent for 5min, rinsed with H_2O, and mounted in glycerine.

Results:

Although the stain will adhere onto the cellwalls and impart a light brown colour to the outline of the cells, it is a stain for starch. Starch stains blue to violet-blue in colour.

Lacmoid - tannic acid - ferric chloride (Cheadle, et al, 1953).

Reagents:

A = 1% aqueous tannic acid

B = 2% aqueous (hydrated) $FeCl_3$

C = 2.5g of $NaHCO_3$ dissolved in 50ml of H_2O

D = 20ml of reagent C diluted with 55ml of H_2O and 25ml of absolute EtOH

E = 0.25g of lacmoid dissolved in 30ml of absolute EtOH and 4ml of reagent C

F = 20ml of reagent C diluted with 30ml of H_2O and 50ml of absolute EtOH

G = equal volumes of absolute EtOH, clove oil and xylol

Procedure:

The tissue sections are immersed in the following reagents:

1. reagent A for 5 - 10min.
2. reagent B for 5min.
3. wash with three changes of H_2O.
4. examine while still wet for wall colour, which should be medium to dark grey. If colour is unsatisfactory, then the above procedures have to be repeated.
5. wash three times in H_2O.
6. immerse in reagent D for 30min.
7. leave in reagent E for 12 to 18hr.
8. reagent F for 10sec to 10min.
9. wash in 80% EtOH.
10. immerse in 90% EtOH for 3min.
11. immerse in 2 changes of absolute EtOH for a total of 3min.
12. wash in reagent G for 3min.
13. wash in 2 changes of xylol for a total of 3min.
14. mount in Canada balsam or DePex.

Results:

For the purposes of taking black - and - white photomicrographs of tissue sections, I find this procedure very satisfactory. Callose stain sky-blue to greenish blue, lignified tissues stain blue, and cellulose, cytoplasm and slime stain light brown to grey brown. The preparations are permanent.

Phloroglucinol

Reagents:

A = 0.1% of phloroglucin in 50% EtOH.

B = concentrated HCl.

Procedure:

Fresh tissue sections are stained in a drop of phloroglucinol for 5min. The excess stain is then blotted away, and a drop of concentrated HCl is added. Sections are examined straight away.

Results:

This is a sensitive test for lignin, which stains red.

General reference for plant histochemical stains

Gurr, E. (1965) — The Rational Use of Dyes in Biology and General Staining Methods.
(Williams and Wilkins Co., Baltimore.)

APPENDIX IV

Some analytical procedures for the study of P.radiata needles

Respiration, with the Warburg apparatus (Umbriet, et al, 1949)

The rate of respiration of fresh P.radiata needle segments was measured in the Warburg respirometer, as indicated in the diagram below.

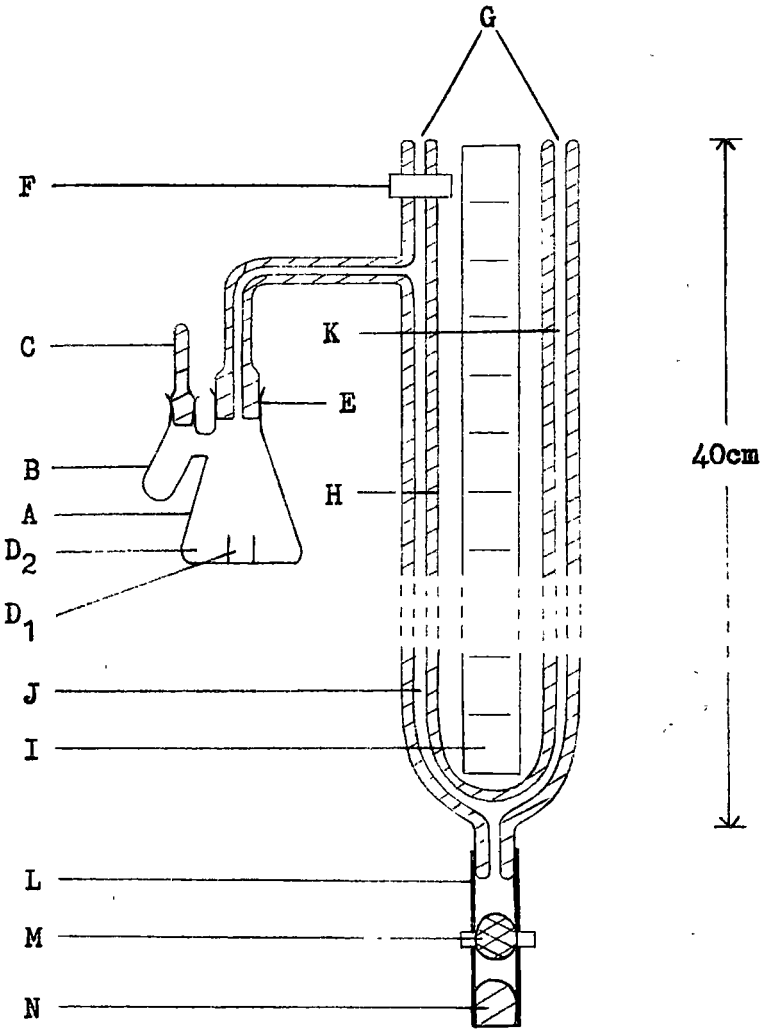

Diagram of the Warburg respirometer

Key for the diagram of the Warburg respirometer

A = Reaction flask

B = Side arm

C = Stopper of side arm

D_1 = Centre well

D_2 = Main compartment

E = Ground glass joint

F = Three way tap

G = Opening of capillary to the atmosphere

H = Constant volume mark on manometer arm

I = Scale, in millimetres

J = Manometer arm, with fluid adjusted to the constant volume mark

K = Manometer arm, indicating pressure changes

L = Rubber tubing, forming the adjustable fluid reservoir. Reservoir is filled with Brodies' fluid.

M = Screw clip, used for adjusting fluid level

N = Glass stopper at the end of the rubber tubing

Umbriet, W.W., Burris, R.H., and Stauffer, J.F. (1949) — "Manometric Techniques in Tissue Metabolism". Revised edition. (Burgress Pub. Co.)

P.radiata needle segments, 1cm long, were placed in the main compartment (D_2) of the flask with 1.0ml of H_2O. In one flask, the centre well (D_1) was left empty but in the other flask, a small piece of filter paper wick placed in D_1 was saturated with 0.3ml of 1M KOH. After ensuring the proper sealing of the ground glass joints with silicone cream, the flasks were immersed in a water bath at 25°C and agitated for 10min. The top of the water bath was also covered with black paper so as to prevent any photosynthesis from taking place. A respirometer with only 1.0ml of H_2O in the main compartment of the flask was also agitated in the water bath, and was used to indicate changes in the atmospheric pressure during the period of experimentation.

After the initial temperature equilibration, the tap (F) was closed so that the arm of the manometer at J was only in contact with the flask A. The fluid level in J was adjusted with the screw clip at M to a constant level H. The height of fluid in the other manometer arm (K) was then recorded. With the capillary at J remaining shut off from the atmosphere, changes to the fluid level at K were measured at 5min intervals. The flasks remained agitated when readings were not taken.

Changes in the fluid level in K give an indication of the changes in air pressure of the respirometer, in the presence of atmospheric pressure. The change in pressure in the manometer was converted to the change in volume at atmospheric pressure, by the following relationship:

$$\text{Volume } (\mu l),\ x = hk, \text{ where } k = \frac{(V_g \times \frac{273}{T}) + (V_f \times \propto T)}{10{,}000}$$

V_g = volume (µl) of gas phase in the respirometer

V_f = volume of liquid contents of the flask

T = temperature, in degrees absolute

αT = solubility of the gas being exchanged in the reaction

At 25°C, α_{O_2} = 0.0283 ml/ml of H_2O at 1 atmosphere

and α_{CO_2} = 0.759 ml/ml of H_2O at 1 atmosphere

The following is an example of a calculation of the results, obtained from actual readings

	Respirometer A	Respirometer B
Volume of H_2O in D_2	1.0ml	1.0ml
Weight of tissues	0.4083g	0.3890g
0.3ml of 1M KOH in D_1	nil	yes
Total volume of flask and manometer, V_T	19.22ml	21.05ml
Volume of fluid and tissues in flask, V_f	1.4083ml	1.6890ml
Volume of gas phase in respirometer, $V_g = V_T - V_f$	17.8117ml	19.3610ml
k_{O_2}	1.6355	1.7782
k_{CO_2}	1.7384	-

For respirometer A,

$$k_{O_2} = \frac{(17.8117 \times \frac{273}{298}) + (1.4083 \times 0.0283)}{10^4 \times 10^{-3}} = 1.6355$$

$$k_{CO_2} = \frac{(17.8117 \times \frac{273}{298}) + (1.4083 \times 0.759)}{} = 1.7384$$

For respirometer B,

$$k_{O_2} = \frac{(19.3610 \times \frac{273}{298}) + (1.6890 \times 0.0283)}{10} = 1.7782$$

Volume of O_2 in respirometer B, $x_{O_2} = H_2 \times k_{O_2}$

$\therefore$ e.g., $x_{O_2} = 5 \times 1.7782$, at time 15min.

$= 8.890$

Volume of CO_2 in respirometer A, $x_{CO_2} = (H_1 - \frac{x_{O_2}}{k_{O_2}}) \times k_{CO_2}$

$\therefore$ e.g., $x_{CO_2} = 0 - (\frac{-1.7780}{1.6355}) \times 1.7384$, at time 5min.

$= 1.8898$

Changes in the pressure of the manometer during respiration, and the corresponding calculated volume changes, are set out in the table below.

		Respirometer A				Respirometer B				
Time (min)	Barometer change	Fluid level h	Δh	Corrected Δh = H_1	x_{CO_2}	h	Δh	H_2	x_{O_2}	RQ
0	-	182	-	-	-	151	-	-	-	-
5	+1	183	+1	0	1.890	151	0	-1	1.778	1.06
10	+2	185	+3	+1	7.513	150	-1	-3	5.334	1.41
15	+2	185	+3	+1	11.188	148	-3	-5	8.890	1.26

Analysis of residues from the cold water extraction of P.radiata needle homogenates

Residues of needle homogenates

Residues were stirred with 4ml of 80% EtOH, and boiled in a water bath for 5min. On cooling, the slurry was centrifuged in a bench centrifuge for 10min and the supernatant was decanted into a test tube. This procedure was repeated twice with 3ml aliquots of 80% EtOH. Supernatants from the three extractions were combined.

Alcoholic supernatant (total volume of 10ml)

Its O.D. was read at 645nm and 663nm against a blank solution consisting of 80% EtOH. The amount of chlorophyll a and chlorophyll b is calculated by the following formula:

$$C_a = (22.9 \times O.D._{645nm}) - (4.68 \times O.D._{663nm}) \text{ mg/ml}$$

$$C_b = (12.7 \times O.D._{663nm}) - (2.69 \times O.D._{645nm}) \text{ mg/ml}$$

Residue from the alcoholic extraction

The procedure for the extraction of "insoluble" protein and RNA was based on the method of Osborne (1962). For this purpose, the residue was washed with each of the following solvents in the order indicated:

1. twice, with 5% trichloracetic acid (i.e., TCA) at 2°C.
2. once, with 80% EtOH.
3. once, with absolute EtOH.
4. twice, with hot EtOH - ether solution (mixed in the ratio of 3 : 1, v/v).

The residue was then suspended in 8ml of 0.3N KOH for 16hr at 37°C. After centrifuging, the supernatant was collected, and the residue was

washed with two 6ml aliquots of H_2O. The combined supernatants, about 20ml, were used for protein measurement, using the Biuret method. A measure of the "insoluble" protein content was thus obtained.

After the extraction of protein from the pellet, the latter was discarded. An aliquot (5ml) of the 0.3N KOH extract was adjusted to pH 1.5 - 2.0 with $HClO_4$. The mixture was centrifuged for 10min in a bench centrifuge, and the volume of the supernatant made up to 20ml by additions from the H_2O washings of the residue. The O.D. of the solution was determined at 260nm against deionised H_2O. This gives a measure of RNA content.

References

Arnon, D.I. (1949) -- Copper enzymes in isolated chloroplasts. Polyphenol oxidase in Beta vulgaris. Plant Physiology, Lancaster, 24, 1 - 15.

Osborne, D.J. (1962) -- Effect of kinetin on protein and nucleic acid metabolism in Xanthium leaves during senescence. Plant Physiology, Lancaster, 37, 595 - 602.

APPENDIX V

t - test for the significance of means and differences of means

[Mather, K. (1966) — Statistical Analysis in Biology. (Methuen & Co., Ltd., London)]

The following is an example of an analysis of means, for the rate of O_2 uptake of P.radiata needle segments, before (i.e., days -21 to 0) and after (i.e., days 7 to 17) mucus treatment.

Rate of O_2 uptake for 50 needle segments

Days before treatment	Rate of O_2 uptake (x_1)	Days after treatment	Rate of O_2 uptake (x_2)
-21	282.91	7	529.67
-14	316.72	9	543.35
-7	236.43	14	506.76
0	242.98	17	527.39
Sum =	1079.04	Sum =	2107.17

x_1	x_1^2	x_2	x_2^2
282.91	80038.0681	529.67	280550.31
316.72	100311.5584	543.35	295229.22
236.43	55899.1449	506.76	256805.70
242.98	59039.2804	527.39	278140.21
1079.04	295288.051	2107.17	1110725.44

	Before treatment	After treatment
Number of observations, n	4	4
Sum of (x)	1079.04	2107.17
Mean of (x), $\bar{x}$	269.76	526.7925
Sum of (x^2)	295288.051	1110725.44
$\frac{\text{Sum}^2 \text{ of } (x)}{n}$	291081.8304	1110036.08
Sum of $(x - \bar{x})^2 = \text{Sum}(x^2) - \frac{\text{Sum}^2 (x)}{n}$	4206.22	689.36
Variance of $x = \frac{\text{Sum of } (x - \bar{x})^2}{N} = V_x$	1402.07	229.787
where $N = n - 1$		
Variance of $\bar{x} = \frac{(\text{Variance of } x)}{n} = V_{\bar{x}}$	350.52	57.447
Standard error of $\bar{x}$, $S_{\bar{x}} = \sqrt{V_{\bar{x}}}$	18.73	7.573

Difference between means, $\bar{d} = \bar{x}_1 - \bar{x}_2 = 257.03$

Variance of $\bar{d}$, $V_{\bar{d}} = V_{\bar{x}_1} - V_{\bar{x}_2} = 407.967$

Standard error of $\bar{d}$, $S_{\bar{d}} = \sqrt{V_{\bar{d}}} = 20.19$

Ratio of deviation to the standard deviation, $t_{(6)} = \frac{\bar{d}}{S_{\bar{d}}}$

$= 12.7305$

Probability of $t_{(6)} = 12.7305$ is less than 0.001

Thus, the change in respiratory rate of P.radiata needle segments following mucus treatment is highly significant.

APPENDIX VI

Electrophoresis

Preparation of polyacrylamide gel for electrophoresis on a semi - micro scale (Mills and Crowden, 1968)

Apparatus for forming the slab of gel:

A perspex wall (Shandon, England) measuring 15 X 9cm in area and 6mm in thickness, was fixed onto a glass sheet by means of vaseline. More vaseline was applied onto the upper surface of the perspex wall. A glass sheet containing a row of fixed perspex teeth (for purposes of forming the gel slots) of dimensions 6 X $5\frac{1}{2}$ X 1 mm, was placed onto the top of the perspex wall, after the gel solution had been poured into the mould. Polymerisation of the gel was allowed to take place at room temperature; during this period of time, the apparatus was left on the level bench, undisturbed.

Gel solution:

To 8g of "Cyanogum" 41 ($\pm$ 0.6g starch powder) was added 100ml of tris - citrate buffer, pH 8.7. The mixture was boiled briefly, and allowed to cool. The catalysts DEAE - cyanide (0.1ml) and ammonium persulphate (0.1g) were then added, and the gel solution was poured into the prepared mould. Care was taken to remove air bubbles which may have had been trapped in the mould.

Buffer solutions:

Gel buffer = 2.5mM citric acid and 38mM tris (tris = tris (hydroxymethyl) amino methane). To make a solution with pH 8.7, about 2l of citric acid (1.0508g/2l) and 1l of tris (4.598g/l) were prepared. The actual proportions of these two solutions, for a pH 8.7 solution, were monitored on a radiometer.

Tank buffer = borate buffer, pH 8.6. This was made up of 15.75g of sodium tetraborate and 7.22g of boric acid, dissolved in one litre of deionised H_2O.

Application of samples, and electrophoretic separation:

The polymerised gel was transferred onto another clean sheet of glass, and excess moisture on the surface of the gel was removed with a piece of tissue paper. A small amount of liquid present in the slots was removed with filter paper wicks.

The gel slots were filled with sample solutions, except for two end slots which were filled with dilute bromophenol blue dye (0.1% aq.). This dye served as an indicator for the extent to which electrophoresis was to be conducted; most electrophoresis experiments were terminated when the bromophenol blue dye had migrated about 5cm from the origin. Strips of muslin saturated with tank buffer were used to form a bridge between the gel and the buffer solution in the electrophoresis tank. A constant current of 34mA was applied for about 3hr, at $5^{\circ}C$.

At the end of the electrophoretic separation, the position of the marker dye was marked by means of puncture holes. The slab of gel was rinsed in water, blotted dry, and then sliced into four with the aid of a Shandon gel slicer. Each of the slices of gel was immediately immersed into trays of stains, for specified time intervals.

Histochemical stains for electrophoretograms

Protein stain

Reagent: 0.7% of Amido black (also known as Amido Schwartz or naphthalene black), in 10% of acetic acid.[a]

Procedure: The slice of gel was immersed in this stain for over-

night, before being washed with warm water until most of the background stain on the electrophoretogram had disappeared.

Results: Protein bands stained black.

Carbohydrate stain, with P.A.S. (Keyser, 1964)

Reagents:

A = 3g of periodic acid (H_5IO_6) and 1.66g of sodium acetate ($NaCOOCH_3.3H_2O$) were dissolved in 500ml of H_2O. For use, 6 volumes of this stock solution was diluted with 4 volumes of 95% EtOH.

B = 2.5g of potassium metabisulphite and 15g of sodium thiosulphate dissolved in 500ml of H_2O. For use, 1 volume of this stock solution was diluted with 1 volume of absolute EtOH.

C = To a solution of 4g of potassium metabisulphite in 500ml of H_2O was added 5.25ml of concentrated HCl, followed by 2g of basic fuchsin. The mixture was stirred for 2hr at room temperature, then stood for 2hr, before being decolourised with 0.5g of decolourising charcoal. The mixture was filtered immediately, and stored in the refrigerator, in a dark bottle.

D = 1.25g of potassium metabisulphite dissolved in 250ml of H_2O. To this solution was added 250ml of EtOH (95%) and 2.25ml of concentrated HCl.

Procedure: The electrophoretogram was immersed in each of these:

1. 95% EtOH for 10min.
2. solution of MeOH : H_2O : HAc (40 : 50 : 5, v/v), for 10min.

3. oxidised in reagent A for 24min.
4. reduced in reagent B for 10min.
5. rinsed with H_2O for 3min.
6. stained in reagent C for 40min.
7. washed in reagent D, with two changes, for 10min. Rewashed in reagent D 30min later, and finally immersed in this reagent for overnight.
8. washed in 0.1N HCl, for 20min.

Results:

Carbohydrates stained magenta in colour.

Amylase stain

Reagent:

2g of iodine dissolved in a solution consisting of 21g of KI in 50ml of H_2O = stock solution.

Procedure:

The electrophoretogram was first immersed in 100ml of buffer solution for 1hr. The buffer solutions were:

a. 0.1M phosphate buffer at pH 6.9, for α-amylase; OR

b. 0.1M acetate buffer at pH 5.0, for β-amylase.

The buffer solution was then decanted off, and the electrophoretogram immersed in a solution of I_2/KI which was made up of 15ml of stock solution and 100ml of the appropriate buffer solution.

Results:

The amylase bands appeared as clear areas on a purplish - blue background.

Substrate for esterase

Reagents:

A = 0.02g of ∝-naphthylacetate dissolved in 1ml of acetone, and mixed with 100ml of phosphate buffer, pH 6.4, 0.1M.

B = 0.04g of Fast Blue BB dissolved in 100ml of phosphate buffer, pH 6.4, 0.1M. Reagent was freshly prepared.

Procedure:

The electrophoretogram was immersed in reagent A for 30min. It was then transferred to reagent B for 1hr. Depending on the concentration of esterase enzyme, the period of incubation in reagent B may have to be prolonged.

Results:

Esterase enzymic bands appeared to be brownish in colour.

Substrate for peroxidase

Reagents:

A = 0.0307g of o-dianisidine dissolved in 5ml of 95% EtOH, with gentle heating. To this was added 95ml of 0.1M acetate buffer, pH 4.5.

B = 30% H_2O_2.

Procedure:

The electrophoretogram was stained in a solution consisting of 2 drops of reagent B and 100ml of reagent A, for 1hr. However, the electrophoretogram was removed from this mixture when the enzymic bands appeared to be too heavily stained during this period of time.

Results:

Peroxidase bands stained brown.

Substrate for phenoloxidase

Reagent:

0.0307g of o-dianisidine dissolved in 5ml of 95% EtOH, with gentle heating. To this was added 95ml of 0.1M acetate buffer, pH 4.5.

Procedure:

The electrophoretogram was stained for about 1hr in the reagent.

Results:

Phenoloxidase bands stained brown.

Numbering of isoenzymes (Wilkinson, 1965)

Two contradictory systems for the numbering of isoenzymes were used by European and American workers. The European system had been adopted by the Standing Committee on Enzymes of the International Union of Biochemistry. This system involved the numbering of isoenzymes in decreasing order of negative charge, so that number 1 was assigned to the isoenzyme with the greatest anodic mobility. A similar numerical convention is adopted here.

APPENDIX VII

Chromatography

Thin layer chromatography

Solvents for sugars:

1. BEW = n-butanol - ethanol - water (4 : 1 : 1, v/v)
2. BPW = n-butanol - pyridine - water (6 : 4 : 3, v/v)
3. EAW = ethylacetate - acetic acid - water (3 : 1 : 1, v/v)

Solvent systems for aminoacids: in combinations of I/II or I/III.

I = n-butanol - acetone - diethylamine - water (40 : 40 : 8 : 2, v/v)

II = sec-butanol - methyl ethyl ketone - dicyclohexylamine - water (40 : 40 : 8 : 20, v/v)

III = isopropanol - formic acid - water (80 : 4 : 20, v/v)

Visualization reagents for chromatograms:

Aminobiphenyl (abbreviated ABP)

Reagents:

1.69g of aminobiphenyl and 0.9g of oxalic acid, dissolved in a solution consisting of 5ml of glycerol, 10ml of H_2O, and 84ml of acetone.

Results:

After heating the sprayed chromatogram for 10min at 100°C, pentoses appeared red, hexoses appeared greenish - brown, and uronic acids appeared purple.

Aniline hydrogen phthalate (abbreviated AHP)

Reagents:

0.93 g of aniline and 1.66g of phthalic acid dissolved in 100ml of water - saturated n-butanol. The reagent was stored in a dark bottle.

Results:

The sprayed chromatogram was heated for 10min at 100°C, to develop the colour of aldopentoses (bright red), aldohexoses, deoxysugars and uronic acids (in shades of green and brown).

Elson - Morgan reagent (abbreviated E-M)

Reagents:

A = 0.2ml of acetylacetone in 20ml of n-butanol

B = 5ml of 50% aqueous KOH (w/v) in 20ml of EtOH

C = a mixture of 20ml of reagent A and 1ml of reagent B

D = 0.2g of p-dimethylaminobenzaldehyde dissolved in 6ml of absolute EtOH and 6ml of concentrated HCl. This solution was diluted with 8ml of n-butanol.

Results:

The chromatogram was first sprayed with reagent C and heated for 1hr at 90°C. After cooling, it was oversprayed with reagent D, and stood at room temperature for 1hr. Glucosamine and galactosamine appeared to be cherry - red; N-acetylated hexosamines appeared to be purple in colour.

Ninhydrin - collidine - acetic acid

Reagents:

0.05g of ninhydrin dissolved in 35ml of absolute EtOH and 10.5ml of acetic acid. To this mixture was added 1.45ml of collidine.

Results:

After heating the sprayed chromatogram for 10min at 100°C, aminoacids appeared in shades of yellow, brown, blue or purple.

Column chromatography

Preparation and regeneration of resins

Biogel (P series)

Initial preparation:

2g of dry Biogel powder was allowed to swell in 200ml of H_2O, with continuous and gentle stirring, for about 12hr. The swollen gel was washed with 400ml of H_2O on a Buchner funnel, and resuspended in 100ml of H_2O. It was then packed into a precision - bore borosilicate column (Pharmacia) of 48ml capacity.

Regeneration:

Gel from the column was emptied into a beaker and suspended in 200ml of H_2O. It was stirred for about 2hr at room temperature, before being washed on a Buchner funnel with about 1 litre of H_2O. The washed gel was packed into the chromatography column and re-used; after which it was discarded.

AG1 - X2 (Cl^- form), 200 - 400 mesh

Initial preparation:

2g of resin was first allowed to swell in H_2O for about 12hr. It was then washed on a Buchner funnel with 500ml of each of the following: 2M NaOH, H_2O, 2M HCl, and twice with H_2O. The resin was resuspended in H_2O and was ready for use.

Regeneration:

The resin was washed on a Buchner funnel with 2 cycles of 500ml aliquots of 2M NaOH, H_2O, 2M HCl, and twice with H_2O. This same resin was regenerated about five times, for use.

AG1 - X2 (Ac^- form), 200 - 400 mesh

Initial preparation:

2g of resin, swollen in water, was washed on a Buchner funnel, with 500ml each of the following: 2M NaOH, H_2O, 2M $NaCOOCH_3$, 0.1M HAc. The resin was resuspended in H_2O and was ready for use.

Regeneration:

The resin was washed on a Buchner funnel with 500ml aliquots of 2M HCl, H_2O, 2M NaOH, H_2O, 2M $NaCOOCH_3$, H_2O; and finally with 2M NaOH, H_2O, 2M $NaCOOCH_3$, 0.1M HAc, and H_2O.

This same resin was regenerated about 5 times, for use.

Dowex 50W - X4 (H^+ form), 200 - 400 mesh

Initial preparation:

2g of resin, swollen in water, was washed on a Buchner funnel with 500ml aliquots of 2M NaOH, H_2O, 2M HCl, and twice with H_2O. The resin was suspended in H_2O and was ready for use.

Regeneration:

The resin was washed on a Buchner funnel with two cycles of 500ml aliquots of 2M NaOH, H_2O, 2M HCl, and twice with H_2O.

This same resin was regenerated about five times, for use.

DEAE - Sephadex (Cl^- form), 200 - 400 mesh

Initial preparation:

1g of resin was swollen in water for about 12hr. It was then

washed on a Buchner funnel with 500ml aliquots of 0.5M NaOH, H_2O, 0.5M HCl, and twice with H_2O. The resin was resuspended in 0.1M of tris - HCl buffer of pH 7.4, and stirred for 2hr at room temperature. The buffer solution was decanted away, and the resin was resuspended in fresh buffer solution and was ready for use.

Regeneration:

For the sake of convenience, fresh resin was used each time. However, regeneration of the resin may be achieved by repeated washing with two cycles of 0.5M NaOH, H_2O, 0.5M HCl, H_2O, and finally with 0.1M tris - HCl buffer, pH 7.4.

Cleansing of contaminated resins

Contamination by microbial growth and chemicals

The resin was poured into a sintered glass funnel of fine porosity, and washed repeatedely with cold 6N HNO_3, until the filtrate was clear. It was then washed thoroughly with H_2O and finally with 2N NaOH until the filtrate was clear. The resin was rewashed with H_2O.

Contamination by proteins

The resin was treated with warm (60°C) 6N HNO_3 and then with warm 2N NaOH, in a manner similar to the treatment of resins contaminated with microbial growth or chemicals.

Contamination by grease

The resin was washed on a sintered glass funnel of fine porosity, with warm (70°C) CCl_4 or acetone, and finally with H_2O.

General references for chromatographic techniques

"Methods in Biochemical Analysis". (Ed. D. Glick.)
Vol. 1. (Interscience: New York.) 1954.

"Methods in Enzymology". (Ed. S.P. Colowick and N.O. Kaplan.)
Vol. 3. (Academic Press Inc., New York.) 1957.

"Methods in Enzymology". (Ed. C.H.W. Hirs.)
Vol. 11. Enzyme Structure.
(Academic Press, New York.) 1967.

APPENDIX VIII

Aminoacid symbols

Symbols for aminoacids are used according to the recommendations of the IUPAC - IUB Commission on biochemical nomenclature symbols for aminoacid derivatives and peptides (1971).

Symbols	Aminoacid	Symbols	Aminoacids
ala	alanine	leu	leucine
arg	arginine	lys	lysine
asp(NH_2) or asn	asparagine	met	methione
asp	aspartic acid	orn	ornithine
cys	cysteine	phe	phenylalanine
glu	glutamic acid	pro	proline
glu(NH_2) or gln	glutamine	ser	serine
gly	glycine	thr	threonine
his	histidine	trp	tryptophan
hyp	hydroxyproline	tyr	tyrosine
Ile	isoleucine	val	valine

APPENDIX IX

Isolation of acid mucopolysaccharides from S.noctilio mucus, with cetylpyridinium chloride (Schiller, et al, 1961)

The following procedure was used for the possible isolation of acid mucopolysaccharides from 1g of autoclaved mucus:

1. 10ml of 10% autoclaved mucus was digested with 0.02g of activated papain for 24hr at 60°C. (In order to activate the enzyme, 0.02g of papain was dissolved in 2ml of 0.1M acetate buffer, pH 5.5, containing 5mM of cysteine-HCl and EDTA, and incubated for 30min at 60°C.)
2. The digest was dialysed against four changes of H_2O for 48hr at 4°C.
3. Protein was precipitated from solution in the presence of 10% TCA, for 6hr at 4°C. The supernatant was collected after centrifugation.
4. Supernatant was dialysed against 2 changes of H_2O for 24hr at 4°C. The volume of the supernatant was then reduced by evaporation.
5. 1g of CPC dissolved in 10ml of 0.04M NaCl was added to the mucus solution. The mixture was incubated at 37°C for 1hr, in order to precipitate the acid mucopolysaccharides (if any).
6. 1g of "heavy" Celite was then added, and the mixture centrifuged for 10min in a bench centrifuge. ("Heavy" Celite was prepared by suspending Celite analytical filter aid (Johns Manville) in H_2O, and decanting off the "fines". The "heavy particles" were dried at 60°C for 12hr, and pulverised.)
7. The supernatant was collected and used for measurements later. The residue was exhaustively extracted with a solution of 0.04M NaCl and 0.1% CPC, and the supernatants obtained from each centrifugation were also set aside for measurements later.

8. The modified carbazole method of Bitter and Muir (1962) was used to monitor the level of hexuronic acid in each of the 0.04M NaCl extracts obtained in steps 5 - 7. When the last extract was free of hexuronic acid, the residue was further exhaustively extracted with a solution of 0.4M NaCl and 0.1% CPC. Hyaluronic acid is soluble in 0.4M NaCl solution.
9. The residue was then further extracted with solutions of 1.2M NaCl and 0.1% CPC (for chondroitin sulphate), and with 2.1M NaCl (for heparin). Each of these extract solutions was also monitored for hexuronic acid level, with the method of Bitter and Muir (1962).
10. The appropriate extract solutions were then pooled together, according to the strength of NaCl present. An excess of 1M KCNS was added to each solution in order to precipitate CPC. Following the addition of "heavy" Celite, the mixtures were centrifuged and the pellets discarded. The supernatants were reduced in volume and dialysed for 48hr at $4^{\circ}C$.
11. The dialysed solutions were fractionated on columns of AG1 - X2 (Cl^{-}). The eluant fractions were monitored for protein and hexuronic acid.

APPENDIX X

Buffer solutions

Acetate buffer, 0.2M

Stock solutions:

A = 0.2M solution of acetic acid

i.e., 11.55ml glacial acetic acid in 1000ml solution

B = 0.2M solution of sodium acetate

i.e., 16.4g of $C_2H_3O_2Na$

OR 27.2g of $C_2H_3O_2Na.3H_2O$ } in 1000ml solution

To make 0.2M of buffer solution, x ml of A + y ml of B.

x	y	pH
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

Carbonate - Bicarbonate buffer, 0.2M

Stock solutions:

A = 0.2M solution of anhydrous sodium carbonate

i.e., 21.2g in 1000ml solution

B = 0.2M solution of sodium bicarbonate

i.e., 16.8g in 1000ml solution

Carbonate - Bicarbonate buffer (continued)

To make 0.2M of buffer solution, x ml of A + y ml of B.

x	y	pH
16.0	34.0	9.6
22.0	28.0	9.8
27.5	22.5	10.0
33.0	17.0	10.2
38.5	11.5	10.4
42.5	7.5	10.6

Phosphate buffer, 0.2M

Stock solutions:

A = 0.2M solution of monobasic sodium phosphate

i.e., 27.8g in 1000ml solution

B = 0.2M solution of dibasic sodium phosphate

i.e., 53.65g of $Na_2HPO_4.7H_2O$

OR 71.7g of $Na_2HPO_4.12H_2O$ in 1000ml solution

To make 0.2M of buffer solution, x ml of A + y ml of B.

x	y	pH
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0

Tris - HCl buffer, 0.1M

Stock solutions:

A = 0.2M solution of tris(hydroxymethyl)aminomethane

i.e., 24.2g in 1000ml solution

B = 0.2M HCl

To make 0.1M of buffer solution, 50 ml of A + x ml of B, diluted to a total volume of 100ml.

x	pH
26.8	8.0
32.5	7.8
38.4	7.6
41.4	7.4
44.2	7.2

Buffer solutions for electrophoresis

Gel buffer = tris-citrate buffer, made from 2.5mM of citric acid (pH 8.7) (1.0508g in 2 litres) and 38mM of tris (4.598g in 1 litre). The exact proportions of citric acid and tris were determined with the aid of a radiometer.

Tank buffer = borate buffer, made up of 15.75g of sodium tetraborate (pH 8.6) and 7.22g of boric acid, dissolved in one litre of H_2O.

General references for buffer solutions

Chayen, J., Bitensky, L., Butcher, R., and Poulter, L. (1969) — A guide to practical histochemistry.
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Mills, A.K., and Crowden, R.K. (1968) -- Distribution of soluble proteins and enzymes during early development of Pisum sativum. Australian Journal Biological Sciences, 21, 1131 - 41.